Characteristics of aggressive B-cell lymphoma

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Chapter 1

Introduction and aim of this thesis

Aggressive B-cell lymphomas

B-cell lymphomas are derived from mature B-cells at various stages of differentiation.[1] In the Netherlands 4,500 patients are diagnosed with a mature B-cell lymphoma annually.[2] Historically B-cell lymphomas have been classified as Hodgkin lymphoma (HL) and non-Hodgkin lymphomas (NHL). The B-NHL encompass a broad clinical spectrum of lymphomas (Figure 1). The WHO 2017 classification recognizes over 30 B-NHL subtypes, with diffuse large B-cell lymphoma (DLBCL) as the most common type in adults.[1] The so called indolent lymphomas, including amongst others follicular lymphoma (FL), marginal zone lymphoma (MZL), mucosa associated lymphoid tissue (MALT) and lymphoplasmacytic lymphoma (LPL), comprise a group of B-NHL that are characterized by a indolent disease course, but with a tendency to relapse. Despite the in general favorable outcome, patients with an indolent lymphoma who relapse early tend to have a poor outcome.[3]

The so-called aggressive B-cell lymphomas make up about 40% of all B-NHL and are characterized by tumor cells with a high proliferation rate. They include DLBCL (37%), primary mediastinal B-cell lymphoma (PBMCL) (3%), high-grade B-cell lymphomas (HGBCL) (3%) and Burkitt lymphoma (BL) (0.8%). Prognosis and treatment of aggressive B-NHL varies depending on type, stage and other biological variables.

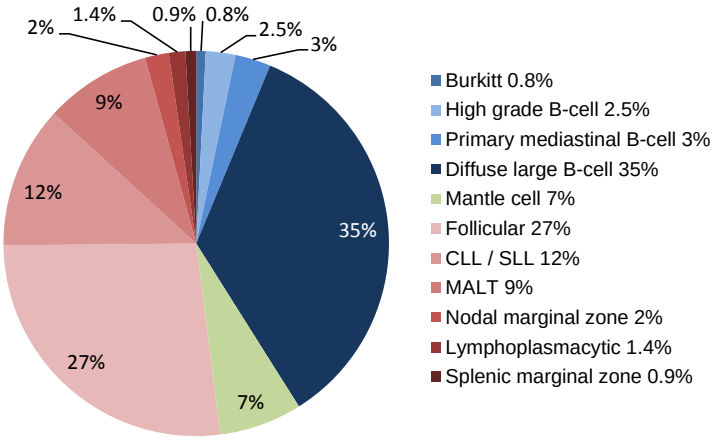

Figure 1: *Relative frequencies of the most common B-cell lymphoma subtypes. The aggressive lymphomas in blue (diffuse large B-cell lymphoma, primary mediastinal B-cell lymphoma, High Grade B-cell lymphoma and Burkitt lymphoma) encompass 40% of B-NHL. The indolent lymphomas are shown in red. Mantle cell lymphoma (shown in green) tends to relapse early. Adapted from Jaffe et al.[1]*

In the WHO 2017 classification there are 14 distinct morphological DLBCL variants.[1] These entities, like primary diffuse large B-cell lymphoma of the CNS (PCNSL), intravascular large B-cell lymphoma and EBV-positive DLBCL have their own unique biological and clinical properties. However, these subtypes are uncommon and the majority of DLBCL cannot be categorized. This unclassifiable DLBCL are referred to as “not otherwise specified (NOS)”.

This thesis will focus on DLBCL-NOS (from here on referred to as DLBCL), and the less common subtypes PMBCL and HGBCL with a *MYC* and *BCL2* and/or *BCL6* rearrangement, often referred to as double hit or triple hit lymphomas (HGBCL-DH). The presentation of all three aggressive B-NHL’s can vary considerably from single lymph node enlargement to life threatening and debilitating disease, e.g. with central nervous system involvement. There are, however, several major clinicopathological differences between DLBCL, HGBCL-DH and PMBCL (Table 1). Especially MYC expression (mostly in relation to rearrangements of the *MYC* locus), expression of the Ki67 proliferative marker and CD30 are very important elements. Clinically, the HGBCL-DH cannot be distinguished from DLBCL, although the majority of patients will present with advanced stage disease and extranodal localization.[1] Morphologically, HGBCL-DH and DLBCL cannot reliably be differentiated from each other. Immunophenotyping for the proliferation marker Ki67 and/or MYC is indicative in some cases, but generally insufficient for classification.[1] Therefore, the diagnostic tool for HGBCL-DH remains fluorescence in situ hybridization (FISH) to detect chromosomal breaks in the *MYC* gene region. Unlike DLBCL, PMBCL’s are most commonly observed in adolescent females presenting with a mediastinal mass, often resulting in a vena cava superior syndrome.[4] PMBCL share several biological features with HL including expression of CD30.[4]

Irrespective of the type of aggressive B-NHL, there is only one opportunity to treat these patients with curative intent, since at relapse these tumors are highly proliferative and long-term remissions can only be achieved in a minority of relapsed patients.[5-9]

Table 1: Major clinicopathological differences between diffuse large B-cell lymphoma, high grade B-cell lymphoma with double or triple hit and primary mediastinal B-cell lymphoma

	DLBCL	**HGBCL-DH**	**PMBCL**
Median Age	65	65	35
Gender	M ≥ F	M ≥ F	Female
Presentation	Nodal / extranodal	Nodal / extranodal	Mediastinal
Stage 1-2 (%)	20	40	90
IHC	CD20+	CD20+	CD20+ / CD30+
Ki67 > 90%	Rare	Common	Rare

Pathobiology

Morphologically DLBCL tumor cells have medium to large aberrant B cells with nuclei equal in size or larger than those of macrophages. The tumor cells show a diffuse growth pattern.
The cell of origin (COO) from which the malignant B cell is derived matters for the prognosis and therapeutic approach. Based on gene expression profiles (GEP) DLBCL-NOS can be subdivided into a germinal center B-cell (GCB) and activated B-cell (ABC) subtype (with ~15% of patients remaining unclassified).[10,11] The two subtypes reflect the B-cell developmental stages from which the tumor cells arise and represent lymphomas with different oncogenic pathways (Figure 2).[12-14]

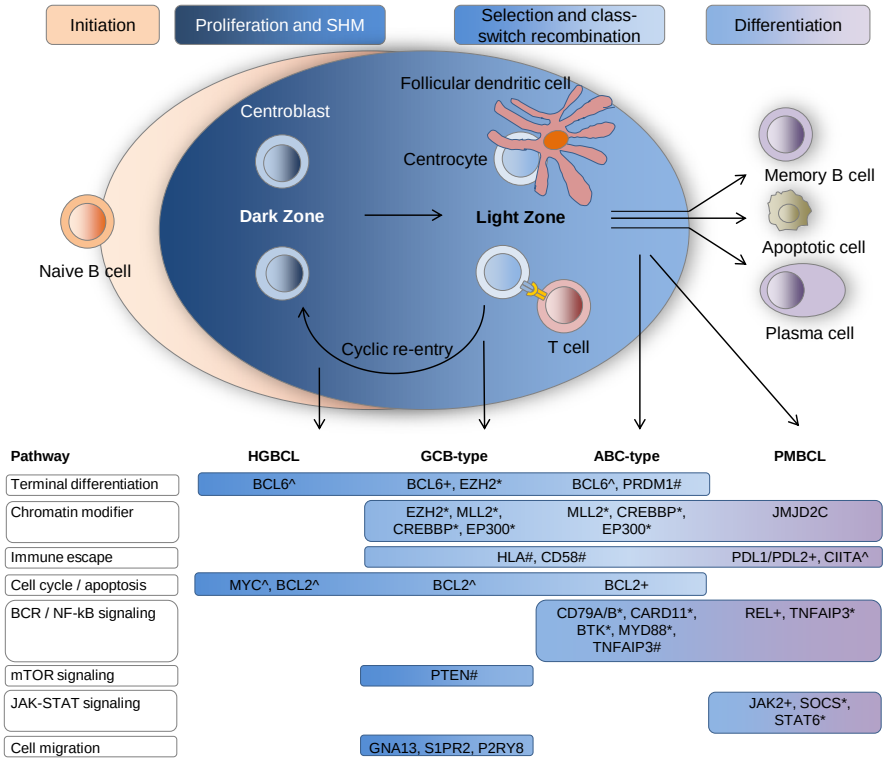

Figure 2: Schematic representation of the developmental stage of lymphomagenesis and key pathways deregulated in diffuse large B-cell lymphoma, High grade B-cell lymphoma with MYC rearrangements and BCL2 and/or BCL6 and primary mediastinal B-cell lymphoma. Legend: * mutation, # loss, ^ translocation, ~ copy number, + expression. Adapted from Basso et al.[14]

Several different transcription factors regulate the transition of B cells through the germinal center reaction. B-cell lymphoma 6 protein (BCL-6) is expressed throughout the germinal center stage. MYC is required for maintaining the B cells in the dark zone (blastoid), whereas NF-kB is expressed during the initation of the germinal center reaction and class switch recombination and PRDM1 is induced in differentiation of plasma cells.[14] Recombination of the Variable, Diversity and Joining (VDJ) gene fragments of the immunoglobulin gene (Ig) is regulated by recombinase-activating genes (*RAG1* and *RAG2*), whereas the somatic hypermutation machinary and class-switch recombination is regulated by activation-induced cytidine deaminase (AID). Although essential for the normal function of the immune system, these processes put B cells at risk of acquiring oncogenic mutations. RAG recombinase can promote translocations involving the immunoglobulin loci, whereas AID can introduce mutations in the non immunoglobulin genes.[15,16]

Irrespective of the COO subtype, DLBCL has several common hallmarks. The majority of DLBCL have an increased expression of the *BCL-6* gene, either by chromosomal translocation affecting the *BCL-6* gene locus at 3q27, activating mutations, loss of IRF-4 repression, gain-of-function mutations in its positive regulator MEF2B and loss-of-function of acetyltransferases CREBPP and EP300. [17,18] Overexpression of BCL-6 abolishes plasma cell differentiation of B cells at the germinal center of maturation by down regulating the master regulator of plasma cell differentiation Blimp-6 / PRDM-1.[19] Secondly, the most frequent inactivating mutations are found in the chromatin modifiers *CREBBP*, *EP300* and *MLL2*, resulting in impaired activation of among other genes the tumor suppressor *TP53*. Third, immune escape through loss of HLA molecules, loss of the cell adhesion molecule CD58 and loss of the co-stimulatory molecule TNFSF9 is observed in > 60% of patients. GEP of DLBCL shows two stromal gene signatures, which are related to prognosis, implicating that the interaction between tumor and immune system shapes the course of the disease.[20]

ABC-subtype

The hallmarks of ABC-type DLBCL are a block in terminal differentiation and activation of the NF-kB signaling pathway. Bi-allelic inactivation of *PRDM1* is observed in 30% of ABC-type DLBCL. Activating mutations in *CD79A/B*, *CARD11* and *MYD88* are observed in 20%, 10% and 35% of cases, respectively. [21-23] These activating mutations result in aberrant NFkB activation through signaling via the B-cell receptor (BCR), CD40 and the toll like receptor (TLR) pathways. Inactivation of TNFAIP3 (A20), which encodes for a negative regulator of the NFkB pathway can be found in 30% of the cases and further contributes to the dependency on activation of the NFkB pathway in ABC-type DLBCL. Activation of the NF-kB pathway prevents apoptosis and causes autocrine signaling of the JAK-STAT signaling pathway by interleukin-6 and interleukin-10.[12]

GCB-subtype

In addition to the generic genetic aberrations in DLBCL, the GCB subtype has several distinctive features. [12-14] Rearrangement of BCL2 occurs in ~40% of GCB-DLBCL. Gain-of-function mutations in the histone methyltransferase EZH2 are present in ~20% of GCB cases. Post germinal center differentiation is blocked due to the repression of CDKN1A, PRDM1 and IRF4. Deletion of PTEN, a tumor suppressor of the PI3K/AKT/mTOR pathway, is present in 10% of GCB-type DLBCL, but loss of expression – as measured by immunohistochemistry - can be observed in upto 55% of the cases.[12-14] Finally, some 30% of GCB-type DLBCL have recently been shown to carry mutations in genes involved in cell migration (*S1PR2, P2RY8, GNA13*).[14] These mutations are thought to enhance B-cell survival outside of the germinal center.

HGBCL-DH

In order to be classified as a HGBCL-DH, these tumors need to have a rearrangement of both the *MYC* gene as well as the *BCL2* and/or *BCL6* genes.[24,25] Given the ~ 40% incidence of *BCL2* rearrangements in the GCB-type, most of the HGBCL-DH have this COO signature. Of note, the majority of MYC rearrangements in HGBCL-DH have non-immunoglobulin partners, like *BCL6*, *ZCCHC7* and *RFTN1*.[25]

Transformed lymphoma

Follicular lymphoma has an annual risk of aggressive transformation of 1-3%, which is associated with an inferior prognosis.[26,27] It includes transformation into various histologies, commonly DLBCL and HGBCL-DH. Recent studies have identified, among other mechanisms, mutations and deletions in *CDKN2A*/*B* and *TP53*.[28,29] *MYC* breaks are present in 25–50% of transformed follicular lymphoma (tFL) and are usually acquired during the process of transformation.[28,29] Hence, tFL harboring a BCL2 and MYC rearrangement can be considered HGBCL-DH.

PMBCL

PMBCL has characteristics of both DLBCL and HL. The tumor cells of PMBCL morphologically resemble those of other large B-cell lymphomas, although multinucleated tumor cells resembling Hodgkin-Reed-Sternberg cells can be observed. Genetically PMBCL has large resemblance with HL, with genetic aberrations of genes involved in immune escape and chromatin modification.[4] Unbalanced rearrangements of *CIITA* (16p13), was observed in 38% of the PMBCL and probably leads to reduced HLA class II expression.[30] In nearly half of patients an amplification of chromosomal region 9p24 is observed, which results in upregulation of the immune checkpoint inhibitors program death ligand 1 and 2 (PDL1-2).[31] Aberrant activation of the JAK-STAT pathway is another hallmark of PMBCL, either through gain of JAK2, mutations of the tumor suppressor gene SOCS1 or mutations of STAT6.[4] Like in ABC-type DLBCL, there is constitutive activation of the NF-kappa-B pathway. Copy number gains of *REL* can be found in 50% of PMBCL, whereas bi-allelic mutations of *TNFAIP3* can be detected in 36% of cases.

Prognosis and risk stratification

Although the prognosis for patients with low risk DLBCL is excellent, the outcome of patients with high risk disease remains unsatisfactory. Apparently, risk stratification is very important. The most frequently used scoring system is the clinical International Prognostic Index (IPI) score, ranging from a 2 year overall survival (OS) of 91% for patients with low risk disease (IPI score 0-1) to 59% for patients with a high risk disease.[32] Besides the IPI score, patients at risk of treatment failure can be identified by tumor localization, amongst which the risk of central nervous system (CNS) recurrence is one of the most common ones [33]. Other prognostic factors that have been reported are interim fludeoxyglucose (^{18}F-FDG) positron emission (PET) scans [34,35], cell-of-origin (COO) [36], MYC translocations [24], MYC/BLC2 expression [37,38], mutations in a high variety of genes [12,39-49], and dynamics of minimal residual disease (MRD), such as reduction of circulating tumor DNA (Figure 3). [42,50,51]

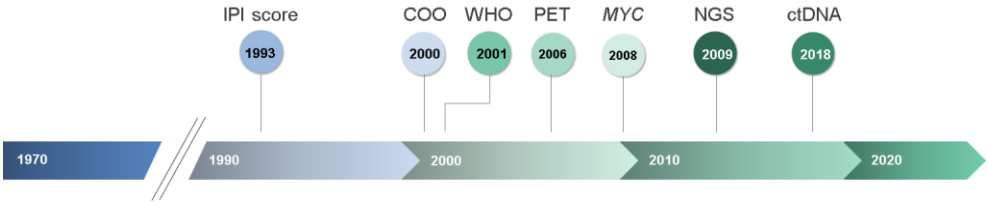

Figure 3: Timeline with diagnostic and prognostic improvements from 1976 until present. Abbreviations: IPI, international prognostic index; COO, cell of origin; WHO, world health organization; PET, positron emission tomography; NGS, next generation sequencing; ctDNA, circulating tumor DNA.

As mentioned above, the initial lymphoma localization is very important for prognosis. For example, patients with a DLBCL of the skin have an excellent outcome after radiotherapy only, whereas patients with CNS involvement (either primary, or secondary) have a poor outcome.[52] It is important to realize that in most scoring systems, patients with these unusual localizations have been excluded.

The IPI-score, a simple and robust scoring model, was first published in 1993.[53] Its prognostic value for aggressive B-cell lymphoma was shown to be valid in the rituximab era.[54] As described before, GEP identified two major DLBCL subgroups, the ABC and GCB type in the year 2000.[10] The ABC-type was shown to have an inferior outcome compared to the GCB-type.[36]

The potential role of ^{18}F-FDG PET-scans in the evaluation of DLBCL was first published in 2005.[55] In 2014 international guidelines were published on the evaluation, staging and response assessment of lymphomas using the 5-point Deauville-scale.[56] Studies on the prognostic value of interim PET-scans, which show good negative predictive value but low positive predictive value, have fuelled various intervention trials using interim ^{18}F-FDG PET-scan in HL (succesfull) and aggressive B-cell lymphomas (unsuccesfull). [34,57-60]

From early 2000 onward the prognostic relevance of MYC rearrangements in aggressive B-cell lymphoma, other than Burkitt lymphoma, was recognized.[24] The HGBCL-DH tend to have a poor response to R-CHOP.[61] Whether the inferior prognosis holds true for the MYC and BCL2 double expressor by immunohistochemistry remains to be established.[37,38]

In recent years, next generation sequencing (NGS) studies have identified various mutations resulting in activation of different pathways (like the NF-kB and mTOR pathway) important for proliferation, survival and protection against cell death.[12,39-49] However, the prognostic and predictive role of the mutational landscape needs to be studied further. Most recently, mutational analysis and assessment of minimal residual disease based on circulating tumor DNA has been introduced as a potential prognostic tool for treatment decision and monitoring.[42,50,51]

Treatment

Until recently the standard treatment for DLBCL, PMBCL and HGBCL-DH patients consisted of 6 to 8 cycles of rituximab combined with cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP). In 1976 cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP) was first introduced for the treatment of aggressive large B-cell lymphomas.[62] Although classification has changed over time, CHOP has been the most commonly used treatment for decades. In patients with limited stage disease 3 CHOP plus involved field radiotherapy (IFRT) was shown to be at least equally effective as 8 cycles of CHOP and subsequently became standard of care in those patients.[63]

The addition of rituximab to CHOP in early 2000 has increased long-term survival of DLBCL patients with 10-15%.[8,9] Rituximab is a human / murine chimeric immunoglobulin G1 monoclonal antibody with specific affinity for the pan-B-cell marker CD20, which eliminates B cells through direct signaling of apoptosis, complement activation and cell-mediated toxicity.[64]

Despite the improved survival of patients treated with R-CHOP, 20% of patients with high risk disease will not achieve a complete remission (CR) after R-CHOP.[9] Patients with a high risk DLBCL, defined as an IPI score > 2, who do achieve CR, have a 2-year progression free survival (PFS) of only 79%.[65] The majority of relapses occur within the first year. Especially patients with these early relapses have a very poor response to current salvage chemotherapy regimens (anti-CD20 antibodies with DHAP, ICE, PECC) and a dismal outcome.[5,6] Only 10-15% of patients with refractory disease or an early relapse (< 12 months after R-CHOP) will achieve a long term remission with the abovementioned salvage regimens.[5-7] Patients with stage I disease are considered to have an excellent outcome, but even amongst those patients relapses occur in about 10-20%.[66] Remarkably, data of the treatment and subsequent outcome of these relapsing patients are scarce.

Central nervous system involvement requires a separate treatment approach. Not only the unfavorable prognosis, but also the blood-brain barrier are reasons for using high dose chemotherapy. High dose regimens with a backbone of methotrexate and cytarabine for patients with primary CNS lymphoma have been studied in several trials and show modest results in patients under the age of 60 year [52,67,68]. The efficacy of high dose chemotherapy in patients with secondary CNS involvement developing at relapse is limited.[69-71] There are no treatment guidelines for patients with the rare occurrence of CNS dissemination at presentation.

During the last decades major advances based upon understanding the pathobiology of mature B-cell lymphomas have resulted in the approval of several novel therapies for the treatment of (relapsed) HL as well as indolent and aggressive B-cell lymphomas (Table 2).

Table 2: Drugs approved for the treatment of mature B-cell lymphomas since 2010

	Line	Drug	Target	Mode	ORR (%)	CR (%)	Ref
FL	R/R	Idelalisib	PI3K	TKI	57	6	[72]
LPL	R/R	Ibrutinib	BTK	TKI	91	73	[73]
MCL	R/R	Lenalidomide	Cereblon	IMID	28	8	[74]
		Ibrutinib	BTK	TKI	68	21	[75]
	First	Bortezomib	Proteasome	PI	OS 91 vs 56 months		[76]
HL	R/R	Brentuximab vedotin	CD30	ADC	72	38	[77]
	First				PFS increase 5%		[78]
	R/R	Pembrolizumab	PD1	CI	69	22	[79]
		Nivolumab			69	16	[80]
DLBCL	R/R	Axicabtagene ciloleucel	CD19	CAR-T	82	54	[81]
	R/R	Tisagenlecleucel	CD19	CAR-T	52	40	[82]

Abbreviations: FL, follicular lymphoma; DLBCL, LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; HL, Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; R/R, relapsed or refractory; PI3K, phosphoinositide 3-kinase; BTK, bruton tyrosine kinase; CD, cluster of differentiation; PD1, programmed death 1; TKI, tyrosine kinase; TKI, tyrosine kinase inhibitor; PI, proteasome inhibitor; ADC, antibody drug conjugate; CI, checkpoint inhibitor; CAR-T, chimeric antigen receptor T-cell; ORR, overall response rate; CR, complete remission; OS, overall survival; PFS, progression free survival. Ref, reference.

Strategies to improve (R)-CHOP can roughly be divided into 4 categories: more intensive chemotherapy, enhanced anti-CD20 therapy, incorporation of novel compounds, and immunotherapy (Figure 4).

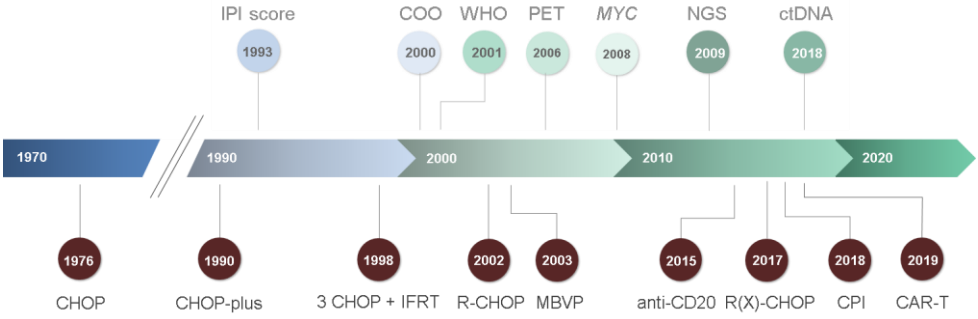

Figure 4: Timeline with attempst to improve treatment in aggressive B-cell lymphomas. Abbreviations: IFRT, involved field radiotherapy; R, rituximab; (X), additional drug; CPI, checkpoint inhibitor; CAR-T, chimeric antigen receptor T cell.

More intensive chemotherapy

In the early '90s more intensive chemotherapy regimens (m-BACOD, ProMACE-CytaBOM and MACOP-B) failed to be superior over CHOP.[83] Although the French showed in a randomized study that ACVBP was superior to CHOP in limited stage DLBCL, this regimen has not been widely adopted.[84] Autologous stem cell transplantation (ASCT) with myeloablative chemotherapy has been studied as a consolidation strategy, but is unlikely to increase overall survival.[85,86] More recently, the more intensive chemo regimens have received renewed interest with the application of dose adjusted EPOCH-R regimen in both PMBCL and HGBCL.[87,88] However, in the setting of DLBCL DA-EPOCH-R does not improve PFS, but increases toxicity.[89] Even more, in the PETAL study, in which patients were escalated to the R-CODOX-M regimen in case of a positive interim PET-scan, there was an increase in treatment related mortality.[34]

Enhanced anti-CD20

Intensifications of rituximab or replacement of rituximab by alternative anti-CD20 monoclonal antibodies have not increased response rates in first line or at relapse.[6,90,91] Until now there are no data supporting the role of rituximab maintenance in DLBCL. The phase 3 NHL13 trial that randomized patients for rituximab maintenance therapy, did not prolong event-free survival (EFS), PFS and OS.[92] Results of the second randomization of the HOVON 84 trial have to be awaited.[91]

Incorporation of novel compounds

Multiple trials have investigated the effect of additional drugs to the R-CHOP regimen, primarily in the ABC subtype. The proteasome inhibitor bortezomib failed to improve response rates.[93] In a phase 3 trial the bruton kinase inhibitor ibrutinib did not improve PFS when combined with R-CHOP.[94] Although in phase 2 trials the immune modulatory agent lenalidomide seemed encouraging, the outcome of the phase 3 trials have to be awaited.[95] Outcome data of the HOVON 130 phase 2 trial which looked at the efficacy of R-CHOP plus lenalidomide in HGBCL-DH looks promising but long-term outcome data have to be awaited.[96] Compounds like the BCL2 inhibitors (NCT02055820) and the antibody drug conjugate polatuzumab-vedotin (NCT01992653) are studied in both the GCB and ABC-type DLBCL. Other studies have employed novel compounds as a consolidation strategy. The phase 3 PILLAR-2 trial did not show an improvement in 2-year DFS for high risk DLBCL (IPI-score > 2) when treated with everolimus consolidation.[97] Lenalidomide maintenance showed encouraging results in relapsed DLBCL.[98] Recent data from a phase 3 trial with lenalidomide consolidation showed a significant improvement in 2-year PFS, unfortunately at the cost of increased toxicity.[99]

Immunotherapy

In the past decade immunotherapy has made major advances in treatment of patients by targeting a series of cell surface molecules known as immune checkpoints.[100.101] The checkpoint molecules can repress the function of pro-inflammatory lymphocytes. Imbalanced activation of signaling pathways, for example through induction of the program death 1 (PD1) ligand, results in altered differentiation profiles and suppression of T cells.[100,101] The monoclonal antibodies against PD1, program death ligand (PDL1) and CTLA-4 have shown substantial therapeutic activity in heavily treated HL patients with also encouraging results in PMBCL.[102-104] In relapsed DLBCL the anti-PD1 antibody nivolumab and the anti-PDL1 antibody atezolizumab showed overall response rates (ORR) of 10% and 27%, respectively.[105,106] A breakthrough for relapsed and refractory DLBCL comes from chimeric antigen receptor T-cells, which target CD19-expressing B cells.[81,82,107] Although these data look very promising with long term remission in 50% of patients with relapsed DLBCL, several major obstacles have to be taken, including bridging therapy, availability, toxicity and costs.

In conclusion, although aggressive B-cell lymphomas DLBCL, HGBCL-DH and PMBCL share morphologic characteristics, there are distinct differences in pathogenesis, clinical presentation, prognosis and treatment. Knowledge on the pathobiology has resulted in the development and approval of several news drugs for their treatment. The last 10-years, the tools for diagnosis, classification and response assessment have greatly improved. However, despite the advances being made on the molecular level, there is a need for better prognostic classification and innovative treatment strategies [108,109].

Scope of this thesis

The aim of this thesis is to investigate what drives aggressive B cell lymphomas (chapter 2-5), to study the characteristics of relapses (chapter 6,7), to explore positron emission tomography (PET) as a diagnostic utitily (chapter 8, 9) and to describe the effectiveness of novel treatments (chapter 10 and 11). In **chapter 2**, we explored which proteins are deregulated in DLBCL compared to Epstein Bar Virus immortalized lymphoblastoid cell lines. In **chapter 3**, we performed a detailed analysis on the role of *MYC* translocations and MYC expression in relation to transformation of FL. In **chapter 4**, we studied a novel mechanism of immune escape in which loss of human leukocyte antigen (HLA) DM expression might result in aberrant membranous invariant chain peptide (CLIP) expression in HLA class II cell surface positive lymphoma cells, preventing presentation of antigenic peptides. Aberrant HLA expression could have implications for the efficacy of checkpoint inhibitors in B-cell lymphomas. In **chapter 5**, we investigated the relation between HLA loss, cell of origin and FoxP1 given the notion that post germinal B-cells downregulate HLA class II when they differentiate into plasma cells. In **chapter 6**, we looked for common characteristics in patients with a limited stage DLBCL who relapse after R-CHOP, since current prognostic models only partially identify patients at risk for relapse. In **chapter 7**, we performend an exploratory study using whole exome sequencing on paired (primary and relapse) DLBCL biopsies to globally assess the mutational evolution and to identify gene mutations specific for relapse samples from patients treated with R-CHOP. In **chapter 8**, we look at the relationship between *MYC* rearrangements and metabolism using fludeoxyglucose (^{18}F-FDG) PET scans in DLBCL and HGBCL-DH, since MYC is the master regulator of metabolism. In **chapter 9**, we describe the impact of false positive ^{18}F-FDG PET scans in PMBCL. In **chapter 10**, we describe the results of the combination of R-CHOP with high dose methotrexate-based chemotherapy in patients with DLBCL with concurrent systemic and central nervous system localization at diagnosis. In **chapter 11**, we describe the succesfull combination therapy of the JAK2 inhitibor ruxolitinib and the program death 1 inhibitor pembrolizumab in a patient with a therapy resistant PMBCL.

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Chapter 2

Proteomics based identification of proteins with deregulated expression in B cell lymphomas

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Abstract

Follicular lymphoma and diffuse large B cell lymphomas comprise the main entities of adult B cell malignancies. Although multiple disease driving gene aberrations have been identified by gene expression and genomic studies, only a few studies focused at the protein level. We applied 2-dimensional gel electrophoresis to compare seven GC B cell non-Hodgkin lymphoma (NHL) cell lines with a lymphoblastoid cell line (LCL). An average of 130 spots were at least two folds different in intensity between NHL cell lines and the LCL. We selected approximately 38 protein spots per NHL cell line and linked them to 145 unique spots based on the location in the gel. 34 spots that were found altered in at least three NHL cell lines when compared to LCL, were submitted for LC-MS/MS. This resulted in 28 unique proteins, a substantial proportion of these proteins were involved in cell motility and cell metabolism. Loss of expression of B2M and gain of expression of PRDX1 and PPIA was confirmed in the cell lines and primary lymphoma tissue. Moreover, inhibition of PPIA with cyclosporine A blocked cell growth of the cell lines, the effect size was associated with the PPIA expression levels. In conclusion, we identified multiple differentially expressed proteins by 2-D proteomics and showed that some of these proteins might play a role in the pathogenesis of NHL.

Introduction

Follicular lymphoma (FL) and diffuse large B cell lymphoma (DLBCL) compose 60% of non-Hodgkin lymphomas (NHLs), both are derived of germinal center or post germinal center B cells [1]. FL is usually an indolent lymphoma, while DLBCL is an aggressive lymphoma [2,3]. Transformation from FL to DLBCL occurs in 25-30% of the patients [4]. Gene expression profiling of DLBCL showed a distinct clustering of cases into two main groups, i.e. germinal center B cell like (GCB) and activated B cell like (ABC) DLBCL [5,6].

To study the transforming mechanisms for germinal B cell derived lymphomas at the protein level several proteomics-based studies have been conducted. The follicular lymphoma derived cell line SUDHL-4 was used to identify secreted proteins [7]. In this study 209 proteins were found with a number of potential candidates for screening, diagnosis and monitoring of treatment efficiency [7]. Mixtures of cell lines were used to perform quantitative analyses by 2-D gel electrophoresis and SILAC approaches [8–10]. Fujii et al [8,9] compared 42 cell lines including Hodgkin lymphoma, B, T and NK cell lymphomas to a reference sample which was a mixture of all cell lines by quantative proteomics. The resulting expression profiles of 389 proteins were used to compare between the different groups of cell lines. Super SILAC was used to compare cell lysates of 5 GCB and 5 ABC DLBCL cell lines using a heavy stable isotype labeled mixture of cell lines as a reference. This yielded a proteome consisting of 7,500 proteins and a subset of 55 proteins that could differentiate between GCB and ABC DLBCL [10]. Comparison of normal B cells, LPS activated B cells and transgenic Eμ-driven murine B cell lymphoma by 2-D gel electrophoresis revealed 48 differentially expressed proteins [11].

In this study we compared the 2-D proteome profiles of NHL cell lines to Epstein Barr virus (EBV) transformed lymphoblastoid cell lines (LCL) to identify differentially expressed proteins. Expression of a selection of the differentially expressed proteins (B2M, PRDX1 and PPIA) was validated in the cell lines and in primary patient material. Inhibition of PPIA with cyclosporine A (CsA) showed a clear effect on cell growth in all NHL cell lines with a correlation between PPIA expression and sensitivity to CsA induced cell death.

Materials and Methods

Cell lines

DOHH2, SUDHL4 (FL), SUDHL6, SUDHL10, OCILY3, Karpas 422 and SUDHL5 (DLBCL) were obtained from DSMZ (Braunschweig, Germany). DOHH2, SUDHL4, OCILY3, Karpas 422 cells were routinely grown at 37°C at 5% CO2 in RPMI 1640 supplemented with 10% fetal calf serum (FCS), ultra-glutamine, penicillin and streptomycin (100U/ml). SUDHL5, SUDHL6 and SUDHL10 cells were cultured with 20% FCS. Five LCLs were generated from peripheral blood mononuclear cells by infection with B95.8 virus. One LCL was used to compare in the 2-D experiments, the other four were used in the validation and functional studies. LCLs were routinely grown in RPMI 1640 with 10% FCS. For the production of LCLs from peripheral blood permission was granted by the Institutional Review board (medical ethical committee UMCG) and written informed consent was obtained.

Patient material

Tissue samples of 46 patients were collected from the pathology biobank for validation by immunohistochemistry. These 46 cases, consisted of 13 low grade FL, 8 FL transformed to DLBCL with evidence of FL in the sample or earlier diagnosis of FL (TFL), and 25 nodal DLBCL. The 25 DLBCL cases were stained for CD10, BCL6 and MUM1 and classified according to the Hans algorithm [12] in GCB (n = 14) and ABC (non-GCB, n = 11). A second group of 137 DLBCL NOS patients, of which 12 patients were also included in the first cohort, was used for the validation of B2M expression. The study protocol was consistent with international ethical and professional guidelines (the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice). The use of anonymous rest material is regulated under the code for good clinical practice in the Netherlands. Informed consent was waived in accordance with Dutch regulations.

Protein extraction

Cells (5–10 x 108) were homogenized in 1 ml of the Homogenize Buffer Mix (BioVision, Milpitas, CA USA) in an ice-cold Dounce homogenizer. The homogenate was centrifuged at 700g for 10 minutes at 4°C. The supernatants were transferred to a new tube and centrifuged at 10,000g for 30 minutes at 4°C. The total cellular membrane protein pellet was lyzed in lysis buffer (8M urea, 4% CHAPS, 2% Pharmalyte) and kept on ice for 30 minutes. The supernatants were harvested by centrifuging at 16,000g for 5 minutes at 4°C. The protein concentrations of the lysates were determined by Bradford assay.

Two-dimensional polyacrylamide gel electrophoresis fractionation of cell extracts

100 µg protein was admixed with rehydration buffer (8M urea, 2% CHAPS, 0.28% dithiothreitol and 0.5% Pharmalyte pH 3–10). Immobilized pH gradient strips (11 cm, pH 3–10) were

rehydrated for 12–16 hours after the protein was loaded. Isoelectric focusing (Bio-Rad, Shanghai, China) was performed at 20°C by the following program: a linear increase from 0–500V over 30 minutes, 500–1000V over 1 hour, 1000–5000V over 4 hours, 5000–8000V 4 hour and then held at 8000V for a total of 64,000Vh. This was followed by a two-step equilibration; first, strips were put into 10 ml equilibration buffer (6M urea, 30% glycerol, 2% SDS, and 50mM Tris-HCl, pH 8.8), which contained 1% dithiothreitol for 15 minutes; next, strips were put into 10 ml equilibration buffer with 2.5% iodoacetamide for 25 minutes, and transferred to 12% SDS polyacrylamide gels. All proteins were visualized by silver staining of the gel, according to standard protocols.

In each experiment, two gels were run in parallel, one with the LCL sample and the second gel with one of the lymphoma cell lines. In order to assure reproducibility, all samples were run at least twice. All gels were scanned with a GS-710 calibrated imaging densitometer imager. The comparative analysis of gels was performed with PD Quest software (BioRad). The density of each spot was evaluated by normalizing volumes of all spots. Spots which were consistently up or downregulated (≥ 2-fold) or spots that appeared or disappeared and were showing consistent differences between LCL and NHL were carefully cut out. For LC-MS/MS spots with the highest density were selected, destained and digested overnight with 5ng/µl trypsin (freshly made in 20mM ammonium bicarbonate pH 8–8.5). After incubation, formic acid was added and gels were incubated 5 minutes on a shaker. They were centrifuged at 5,000rpm for 1 minute and the supernatant was collected for LC-MS/MS analysis with the LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany).

Protein identification

The peaks and sequences of peptides from selected protein spots were identified by ProteinPilot 3.0 (Applied Biosystems). Proteins were identified by using the UniprotKB/Swiss-Prot database [13]. Proteins with the correct molecular weight and the highest peptide coverage were considered as the correct protein.

Flow cytometry

Cells were collected by centrifuging at 1200rpm for 5 minutes at 4°C and incubated with an anti-B2M antibody (1:750, Dako, Glostrup, Denmark) for 30 minutes on ice. Cells were washed with 1ml 1% PBS/BSA and FITC labeled goat anti rabbit antibody (1:10, Southern Biotech, Birmingham, AL USA) was added as the secondary antibody. Acquisition was performed on a Calibur flow cytometer (BD Biosciences, San Jose, CA USA) and data were analyzed with Winlist software.

Quantitative RT-PCR

Total RNA was isolated using QIAzol (Carlsbad, CA USA) and samples were DNAse treated (Ambion, Foster City, CA USA) according to the manufacturer's protocol for cell lines. RNA concentration was quantified using the Nanodrop™ 1000 Spectrophotometer (Thermo Fisher

Scientific Inc., Waltham, MA USA) and RNA integrity was evaluated by 1% agarose electrophoresis. cDNA was synthesized using 500ng input RNA, Superscript II and random primer according to the manufacturer's protocol (Invitrogen, Bleiswijk, the Netherlands). Primers used were for PRDX1 forward 5'-AGCCTGTCTGACTACAAAGGAAAATAT-3' and reverse 5'-GGCACACAAAGGTGAAGTCAAG-3' and for PPIA forward 5'-GCTGTTTGCAGACAAGGTCC-3' and reverse 5'-CAGGAACCCTTATAACCAAATCC-3'. The qPCR reaction was performed in triplicate in a final volume of 10µl consisting of 5µl SYBR Green mix (Applied Biosystems, Foster City, CA USA), 2µl of forward and reverse primer (300mM) and 2,5µl 1ng of cDNA. Amplification was performed on a Roche LightCycler1 480 Instrument (Roche, Almere, the Netherlands). TBP was used as a housekeeping gene and 2-ΔCp values were calculated.

Immunohistochemistry

Immunohistochemistry was performed according to standard protocols with appropriate positive and negative controls. Antibodies used were: anti-B2M (1:200, antigen retrieval with TRIS/EDTA pH9, Dako), anti-PRDX1 (1:200, antigen retrieval with citrate buffer pH6, Abcam, Cambridge, UK) and anti-PPIA (1:800, antigen retrieval with citrate buffer pH6, Abcam).

Cytotoxicity assay

Cell lines were cultured in triplicate at 105 cells/ml with different concentrations (0–10µg/ml) of Cyclosporine A and Alamar Blue (Abd Serotec, Oxford, UK). Cultures were measured every 24 hours for 3 days at an emission of 560nm and extinction of 590nm. Experiments were performed 3 times.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 22. The Mann-Whitney U-test was used to compare B2M, PRDX1, and PPIA expression levels in NHL groups and LCLs for MFI and mRNA levels. Differences of B2M, PRDX1, and PPIA staining were defined by Chi-square test for immunohistochemistry. A paired T-test was performed to define the difference in cell viability before and after cyclosporine A treatment. The correlation of PPIA expression level and cell viability was defined by Spearman-test. All analyses were two-tailed. $P<0.05$ was considered as significant.

Results

Proteome profiles of LCL and NHL cell lines and protein identification

An average of 1,133 (±355) and 1,119 (±330) spots were detected in the 2-D gels of the LCL and NHL cell lines, respectively (Figure A in S1 File). The paired match rate of spots on control gels to lymphoma cell line gels ranged from 92 to 96% indicating a good consistency. We excluded spots that were too weak in both gels or that were incorrectly annotated. This resulted in an average of 248 (±24) reliable spots per gel that could be used for differential expression analysis. An average of 130 (±25) spots were at least 2-fold up or downregulated between the paired NHL and LCL cell lines. We picked 38 (±4) protein spots in each pair of gels, based on sufficiently high expression levels to analyse and reliable separation on the 2-D gel. Based on the position in the 2-D gels we were able to link them to 145 unique protein spots. Of these 34 spots were found in at least three NHL cell lines. Spots that were consistently up or downregulated (n = 22) were pooled for protein identification, whereas spots that were up in some and downregulated in other NHL cell lines (n = 12 spots, resulting in 2x 12 protein IDs) were analyzed separately. For the 34 spots differentially expressed in 3 or more cell lines a total of 46 analyses were performed. The results are summarized in Table 1. Of the 12 spots that were analyzed in duplicate and were upregulated in some and downregulated in other cell lines, 7 represented the same protein, while 5 represented different proteins with similar molecular weights. Of those 5 spots (10 different proteins) only 4 were found in at least 3 cell lines, the other 6 were removed from further analysis. Four proteins were found twice (PFN1, CFL1, PRDX1 and PPIA) at similar weight but at different iso-electric focusing points, probably due to posttranslational modifications such as phosphorylation, and are indicated as modified.

Fourteen proteins, i.e. B2M, FAHD1, PRDX4, LYZ, CALM1, ARPC5, CALR, TUBB, PRDX3, RPSA, ATIC, RPS12, PFN1, and CFL1, were downregulated in NHL cell lines compared to LCL cell lines and 8 proteins, i.e. CFL1 (modified), PPIA (modified), MDH2, PRDX1 (modified), MDH1, ENO1, PRDX2 and PCBP1 were upregulated (Fig 1). The remaining 10 proteins, i.e. LGALS1, PFN1 (modified), MYL6, SSBP1, CAPZA1, GSTP1, IDH3A, PPIA, PRDX1 and PKM, were downregulated in some of the NHL cell lines and upregulated in others. The identified proteins are involved in cell motility (n = 6), cell metabolism (n = 5), chromatin modification and transcription (n = 5), anti-oxidant (n = 4), immune response (n = 4), signal transduction and membrane transport (n = 3) and drug metabolism (n = 1) (Fig 1).

We selected B2M, PRDX1 and PPIA for further validation based on the differential expression patterns in the NHL cell lines and availability of suitable antibodies for immunohistochemistry. B2M plays a role in the immune response, PRDX1 has an anti-oxidant function, and PPIA is involved in signal transduction.

Validation of B2M

Expression of B2M was absent or reduced in all NHL cell lines compared to the LCL cell line in the 2-D analysis (Fig 2A). These 2-D results were validated on the cell lines by flow cytometry (Fig 2D). Four of the NHL cell lines showed lower mean fluorescent intensity (MFI) as compared to the 4 LCLs, consistent with the 2-D analysis, whereas the other three cell lines showed a similar MFI. Immunohistochemistry of 46 primary cases revealed total loss of B2M expression in 21% of cases, i.e. 1 out of 13 FL, 2 out of 8 TFL, 5 out of 14 GCB, and 2 out of 11 ABC. In addition, 9% of the patients showed cytoplasmic staining for B2M, i.e. 1 TFL, 1 GCB, 3 ABC (Fig 3A–3C), so in total 30% of cases showed no membrane expression of B2M. Since B2M loss was most common in DLBCL patients, we further checked loss of B2M expression in a larger cohort of 137 DLBCL patients. Of the 126 evaluable cases 35 (28%) were completely negative for B2M while 29 patients (23%) showed cytoplasmic expression of B2M, so a loss of membrane B2M expression was observed in a total of 51% of the DLBCL cases.

Validation of PRDX1

PRDX1 (modified) was upregulated in 3 NHL cell lines (SUDHL4, SUDHL6 and SUDHL10) compared to the LCL cell line. Expression of PRDX1 was upregulated in 2 NHL cell lines (SUDHL4 and SUDHL10) and downregulated in 1 NHL cell line (SUDHL5)(Fig 2B). PRDX1 mRNA expression levels were higher in SUDHL4, SUDHL10 and SUDHL6 (Fig 2E) compared to the LCL cell lines, consistent with the 2-D results. In contrast, SUDHL5 had the highest mRNA levels, while in the 2-D experiment PRDX1 protein levels were downregulated compared to the LCL cell line. Immunohistochemistry of the 46 primary NHL cases showed positive staining in 33 cases (72%). In FL, 5 of the 13 cases were positive with 4 cases showing weak positive and 1 case showing strong positive staining. Of the TFL cases, 3 showed weak positive staining and 3 strong staining. In GCB DLBCL 10 cases were weak positive and 3 cases were strong positive. Of the ABC DLBCL cases 5 showed weak positive staining and 4 showed strong staining (Fig 3D–3F). When comparing the staining pattern of PRDX1 in the NHL subtypes, a significant difference ($p = 0.0409$) was found. Comparison of the staining results between the three lymphoma subtypes revealed a significant difference between FL and GCB-DLBCL ($p = 0.0112$).

Validation of PPIA

The expression of PPIA (modified) was upregulated in 4 cell lines (DOHH2, SUDHL4, OCILY3 and Karpas 422) compared to LCL in the 2-D gels. The unmodified PPIA was upregulated in 2 cell lines (DOHH2 and SUDHL4) and downregulated in 1 cell line (SUDHL6) (Fig 2C).

PPIA mRNA expression levels were upregulated in all NHL cell lines compared to LCLs ($p = 0.0040$, Fig 2F). SUDHL4 mRNA levels were highest, fitting the 2-D pattern, while the other NHL cell lines with upregulated protein levels in the 2-D (DOHH2, OCILY3 and Karpas 422) analysis were amongst the lowest at the mRNA levels. Immunohistochemistry of primary cases revealed in

most of the FL cases (8 of 13) weak positive staining, while all 8 TFL cases showed strong positive staining for PPIA. GCB and ABC DLBCL staining results were comparable with 9 and 7 strong positive cases, for ABC the remaining cases were weak positive, while for GCB they were partly weak and partly negative. The results are summarized in Fig 3G–3I. The pattern of PPIA expression in primary patient material was significantly different ($p = 0.0418$) with the significant difference between FL and TFL ($p = 0.0079$).

To further investigate the role of PPIA in NHL, we inhibited PPIA in all cell lines with cyclosporine A (CsA). After treating the cells for 72 hours with different concentrations (0, 0.5, 1, 2, 5, 10 μg/ml) of CsA the viability of LCL and NHL cell lines was assessed (Fig 4; Figure B in S1 File). The NHL cell lines were significantly more sensitive to the effect of CsA than the LCL cell lines at each concentration. SUDHL5 and SUDHL10 were most sensitive. To explore whether the expression level of PPIA is related to the sensitivity of CsA treatment, we correlated the PPIA mRNA level with the relative cell viability of 7 NHL and 4 LCL cell lines upon treatment with CsA. A significant negative correlation was observed between PPIA mRNA level and the viability of cells after CsA treatment, with the most significant effect in cells treated with 5 μg/ml CsA ($p = 0.0064$, $r2 = 0.6112$; Fig 4; Figure B in S1 File).

Discussion

Our 2-D gel electrophoresis approach revealed 28 differentially expressed proteins in lymphoma cell lines compared to LCLs. LCLs were chosen since they are cell lines and have similar proliferation patterns, while normal B cells are in a resting stage. For LCLs the transformation mechanism is by EBV and the proteins involved should be different from those in the NHL cell lines. The identified proteins are involved in various processes relevant to the pathogenesis of B cell lymphoma including cell motility and metabolism.

The proteins included in the cell motility gene ontology are ARPC5, TUBB, CFL1, MYL6, CAPZA1 and PFN1. With the exception of the modified CFL1 and PFN1, these proteins are all downregulated in NHL. ARPC5, CFL1, CAPZA1 and PFN1 have been shown to play roles in metastasis and invasion of solid tumors. Downregulation of ARPC5 blocks metastasis [14], whereas downregulation of CAPZA1 [15] and PFN1 [16] enhances metastasis and invasion or motility of cells. The presence of phosphorylated CFL1 [17] is associated with metastasis, and phosphorylated PFN1 [18] leads to enhanced angiogenesis via the upregulation of HIF1α. The functional consequences of downregulation of this group of proteins in NHL remains unknown. The proteins associated with metabolism are involved in the process known as the Warburg effect [19], which is a hallmark of cancer. PKM and ENO-1 are part of glycolysis, while IDH3, MDH1 and

MDH2 are part of the Krebs cycle. Four of the five metabolism proteins are upregulated in NHL, except for IDH3 which was downregulated in 2 cell lines and upregulated in one. The PRDX proteins (anti oxidative proteins) are indirectly related to the Warburg effect, since reactive oxygen species levels are up as a result of the Warburg effect and the PRDX proteins can neutralize that effect. Upregulation of PRDX1 and PRDX2 fits with the upregulation of metabolism proteins, however, PRDX3 and PRDX4 were downregulated in NHL compared to LCL cell lines.
Various proteins identified by us as differentially expressed in NHL compared to LCL have been identified in B cell lymphoma previously. In the Eμ-driven mouse model Romesser et al found differential expression of ENO1, CALR, CFL1 and PPIA by comparing the proteome of activated B cells to B cell lymphoma [11] Consistent with their results we found downregulation of CALR and upregulation of the modified CFL1 protein and PPIA in lymphoma compared to LCL cells. The unmodified CFL1 protein was downregulated in our experiments, suggesting a shift towards the modified form of the CFL1 protein, which could indicate a more active form due to for example phosphorylation. PRDX1 expression was reported in a LCL cell line [20] and in DLBCL [21]. We found upregulation of PRDX1 in NHL compared to LCL. PRDX4 was shown to be upregulated in DLBCL [21], while we found downregulation in NHL compared to LCL. Protein levels of GSTP1 were reported to be high in 29% of DLBCL cases and low in all FL cases [22] Expression of GSTP1 has also been found with 2-D electrophoresis in a LCL cell line [23]. In our study, GSTP1 was downregulated in 2 cell lines and upregulated in one cell line. Loss of B2M has been described previously in DLBCL of testis and the central nervous system as well as in DLBCL NOS [24,25].

B2M levels were decreased in all seven NHL cell lines compared to LCL. LGALS1 expression has been reported in 7% of DLBCL [26]. In LCLs and EBV+ post-transplant lymphoproliferative disorders expression of LGALS1 has been reported upregulated [27]. We found downregulation in 3 cell lines and upregulation in 1 cell line, comparable to the reported data. Thus 9 of 28 proteins identified in this study have been reported in B cell lymphoma before and showed expression changes consistent with the literature. B2M, expression is downregulated or absent in all NHL cell lines compared to LCL cells. This observation is partially supported by flow cytometry and IHC staining. B2M is one of the polypeptide chains of human leukocyte antigen class I (HLA class I), and both chains are essential for membrane expression of HLA class I. Loss of HLA class I expression provides an immune escape mechanism for tumor cells. A number of studies have shown loss of HLA class I in DLBCL patients, especially in DLBCL presenting at immune privileged sites [28]. Loss of B2M membrane expression has been published in up to 60% of DLBCL cases [25]. In our DLBCL cohort we found loss of B2M membrane expression in 51% of cases.

Another differentially expressed protein we identified, CALR, has also been associated with antigen presentation [29]. CALR expression is decreased or lost in some NHL cell lines compared to LCLs. CALR is a chaperone molecule for HLA class I stability, as well as a chaperone for misfolded proteins [29]. Loss of CALR might be part of the immune escape mechanism or alternatively be a consequence of the loss of HLA class I expression.

PRDX1 expression is elevated at protein and mRNA level in part of NHL cell lines compared to LCL cells. In patient samples, we observed a higher proportion of strong positive and positive PRDX1 expression in the more aggressive TFL and DLBCL as compared to the indolent FL, this difference is most pronounced in the comparison between GCB-DLBCL and FL. Peroxiredoxins are thiol peroxidases, that play a role in maintaining the redox balance and have anti-apoptotic ability. PRDX1 is expressed in germinal center B cells and plasma cells and in germinal center derived B cell lymphomas and multiple myeloma [21]. The expression of PRDX1 in NHL could play a role in protection against apoptosis or be part of the Warburg effect.

PPIA, expression was elevated in the 2-D in 3 cell lines and mRNA levels are consistently higher in NHL cell lines compared to LCL cells. In addition, PPIA protein expression is higher in aggressive TFL and DLBCL as compared to the indolent FL. PPIA belongs to the immunophilin family, which catalyzes cis-trans isomerization during protein folding. Elevation of PPIA expression has been observed in some solid cancers, such as non-small cell lung carcinoma [30] and gastric cancer [31]. Knockdown of PPIA can inhibit growth of non-small cell lung carcinoma cells [32]. PPIA supports neoplastic cell proliferation, cell cycle progression and invasion, while protecting them from apoptosis, by activating signaling pathways, such as NF-κB [33], and ERK1/2 [34]. PPIA can also be secreted into the circulation to attract monocytes and stimulates monocytes to produce IL-6 [35,36], and thereby create a pro-tumor microenvironment.

Cyclosporin A (CsA), a widely used immune suppressive drug, binds to PPIA and inhibits its function. In T cells, CsA inhibits transcription factor NF-AT which is important in activation of T cells. In our study, there is a correlation between PPIA mRNA level and CsA induced cell death. The lymphoma cell lines showed significant more inhibition of cell growth than the LCLs, at all tested CsA levels. CsA suppressed tumor progression of squamous cell carcinoma and murine B cell lymphoma in a mouse model [37]. In mouse xenograft models for bladder cancer [38] and breast cancer. [39] CsA treatment prevented tumor growth. The use of CsA in patients has been tested in angioblastic T cell lymphoma, with a response to therapy in 8 out of 12 patients [40]. These data support CsA treatment as a novel therapy in PPIA positive DLBCL. However, the induction of EBV+ lymphoproliferative disease is a severe and well known side effect of long-term immune suppression by CsA in the post-transplantation setting.

Conclusion

In summary, we identified 28 differentially expressed proteins and validated the expression in primary tissue samples for three selected proteins. The finding of B2M and PRDX1 confirmed validity of our approach as it has been shown previously in NHL. Elevated PPIA expression in lymphoma compared with LCL cells is novel and its oncogenic potential is supported by the inhibition of cell growth upon CsA treatment.

Tables

Table 1: Altered expression of proteins identified by LC-MS/MS

spot number	protein name	UniProtKB/Swiss-Prot	Mass (kDa)	Sequence coverage (%)
1	RPSA	P08865	32.854	64
2	TUBB	P07437	49.671	45
3	PRDX1*	Q06830	22.11	76
4	PRDX3	P30048	27.693	59
5	CALR	P27797	48.142	67
6	PRDX2	P32119	21.892	79
7	MDH1	P40925	36.426	57
8	ARPC5	O15511	16.32	77
9	PFN1	P07737	15.054	74
10	CALM1	P62158	16.838	48
11	LYZ	P61626	16.537	18
12	RPS12	P25398	14.515	76
13	ATIC	P31939	64.616	66
14	PCBP1	Q15365	37.498	67
15	ENO1	P06733	47.169	47
16A	PRDX4	Q13162	30.54	10
16B	TPI1	P60174	30.791	60
17A	EEF1B2	P24534	24.764	70
17B	NUDT5	Q9UKK9	24.328	19
18	CFL1	P23528	18.502	73
19A	LGALS1	P09382	14.716	81
19B	LGALS1	P09382	14.716	73
20	PPIA*	P62937	18.012	75
21A	PFN1*	P07737	15.054	69
21B	PFN1*	P07737	15.054	84
22A	ECHS1	P30084	31.387	72
22B	ACO2	Q99798	85.425	54
23A	GNB2L1	P63244	35.077	18
23B	MDH2	P40926	35.503	51
24A	FAHD1	Q6P587	24.843	37
24B	HSD17B10	Q99714	26.923	26
25	CFL1*	P23528	18.502	83
26A	PKM	P14618	57.937	69
26B	PKM	P14618	57.937	4
27	B2M	P61769	13.715	71
28A	MYL6	P60660	16.961	63
28B	MYL6	P60660	16.961	26
29	SSBP1	Q04837	17.260	27
30A	CAPZA1	P572907	32.923	57
30B	CAPZA1	P572907	32.923	26
31A	GSTP1	P09211	23.356	32
31B	GSTP1	P09211	23.356	68
32A	IDH3A	P50213	39.592	27
32B	IDH3A	P50213	39.592	29
33	PRDX1	Q06830	22.110	53
34	PPIA	P62937	18.012	82

Figures

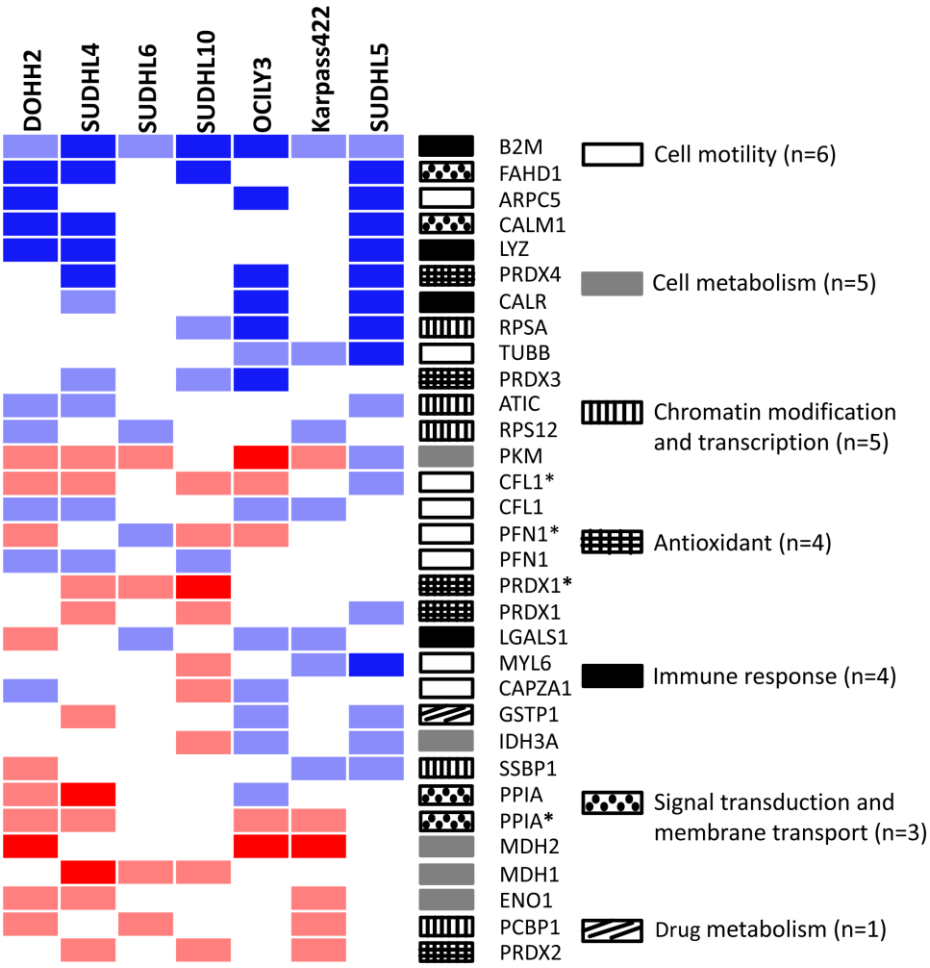


Figure 1. Heatmap of 2-D spots with different intensities in NHL compared with the LCL cell line. Proteins that were differentially expressed in at least 3 cell lines are shown. In total, 28 different proteins have been found and 4 proteins were also found in a modified form (*). Blue: protein spot is missing in NHL cell lines. Light blue: protein spot is down-regulated in NHL cell lines. Red: protein spot is missing in LCLs and thus increased in NHL cell lines. Light red: Protein spot is weaker in LCL and thus elevated in NHL cell lines. Gene ontology was checked to classify the proteins according to function.

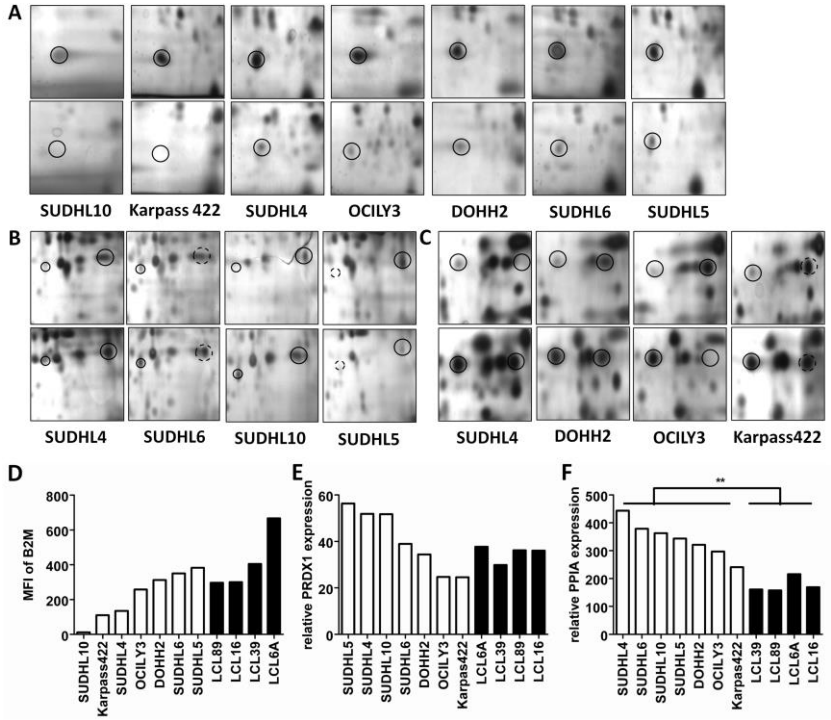


Figure 2. Expression of B2M, PRDX1 and PPIA. (A-C) Silver stained 2 Dimensional SDS PAGE gels. Differential expressed spots have been circled. Continuous circles indicate differential expression was more than 2-fold. Dotted circles indicate difference in expression level was less than 2-fold. Upper line pictures are LCL and lower lines are NHL cell lines. (A) SUDHL10, Karpas 422, SUDHL4, OCILY3, DOHH2, SUDHL6 and SUDHL5 are shown. Loss of B2M is observed in SUDHL10 and Karpas 422, decreased expression of B2M is seen in SUDHL4, OCILY3 and DOHH2, and moderate decreased expression of B2M is seen in SUDHL6 and SUDHL5 compared to LCL. (B) SUDHL4, SUDHL6, SUDHL10 and SUDHL5 are shown. Modified proteins are located on the left. Elevated expression of PRDX1 is observed in SUDHL4, SUDHL10 and decreased expression of PRDX1 in SUDHL5 compared to LCL. Elevated expression of modified PRDX1 is observed in SUDHL4, SUDHL10 and SUDHL5 compared to LCL. (C) SUDHL4, DOHH2, Karpas 422 and OCILY3 are shown. Modified proteins are located on the left. Elevated expression of PPIA is observed in SUDHL4, DOHH2 and decreased expression of PPIA in OCILY3 compared to LCL. Elevated expression of modified PPIA is observed in SUDHL4, DOHH2, OCILY3 and Karpas 422 compared to LCL. (D) The mean fluorescent intensity of B2M expression determined by flow cytometry. (E) The mRNA expression level of PRDX1. (F) The mRNA expression level of PPIA. Mann-Whitney U test was used to compare the differences in B2M, PRDX1, and PPIA expression between the NHL cell line group and LCL group (**:$p<0.01$).

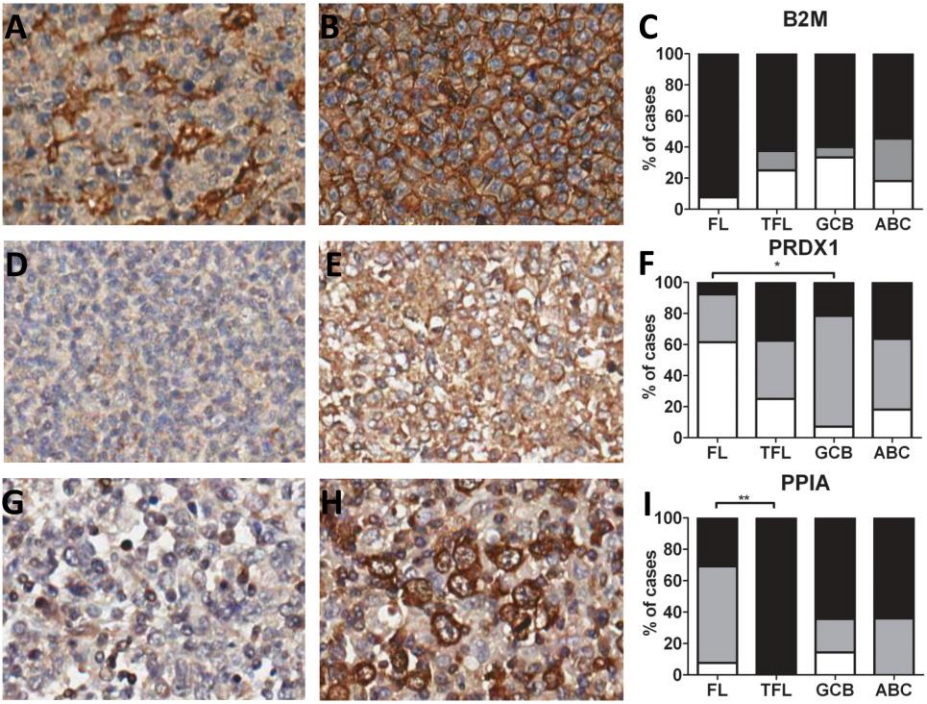


Figure 3. Immunohistochemistry staining of B2M, PRDX1 and PPIA. (A-C) B2M, (D-F) PRDX1, (G-I) PPIA. (A, D, G) negative cases, (B, E, H) positive cases. (C, F, I) Percentage of positive cases in different types of NHLs. Black bar indicates positive staining; grey bar indicates cytoplasmic staining for B2M and weak staining for PRDX1 and PPIA; white bar indicates negative staining. Chi-square test were performed to determine the differences in the B2M, PRDX1, and PPIA staining between the NHL subtypes (*: $p<0.05$; **: $p< 0.01$).

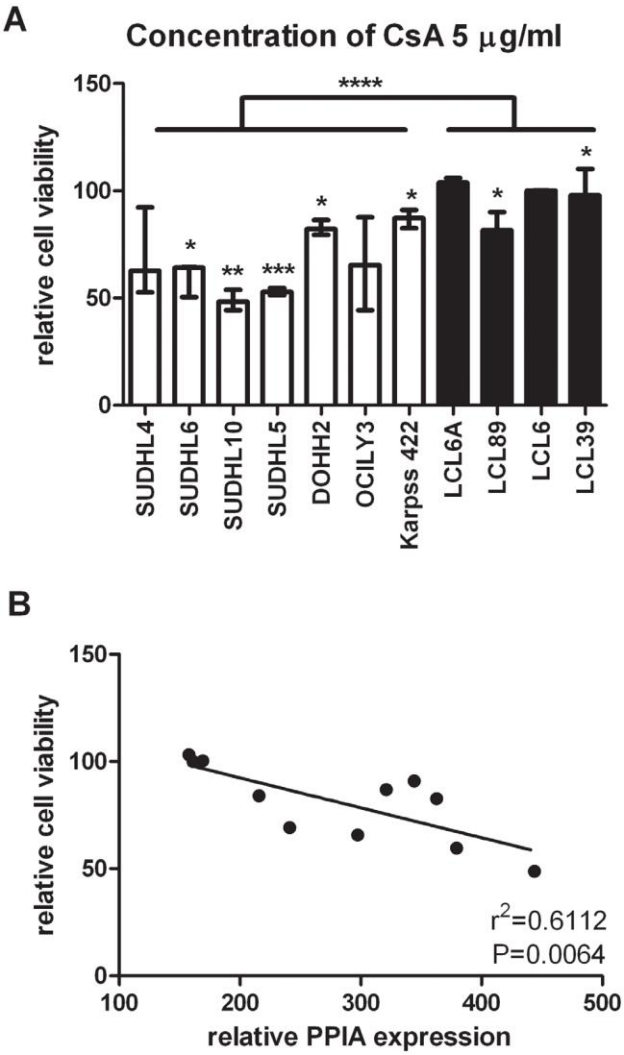
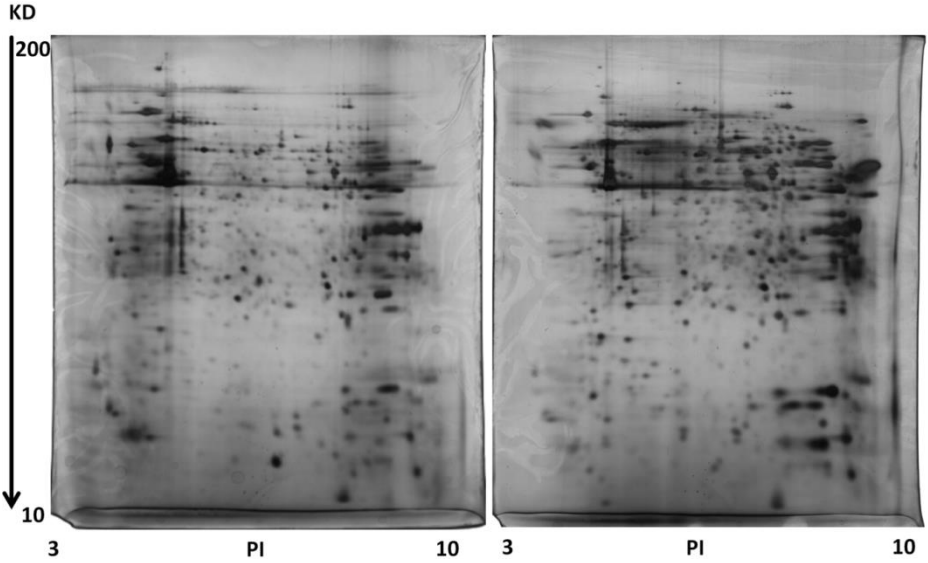


Figure 4. Sensitivity of NHL and LCL cell lines to inhibition of PPIA by 5 μg/ml CsA. (A) The inhibition of cell viability after CsA treatment. After adding 5 μg/ml CsA for 72 hours, the viability of NHL and LCL cell lines was evaluated by alamar blue assay. A paired T-test was performed to compare the cell viability before and after treatment. Mann-Whitney U test was used to compare the cell viability between the NHL cell line group and LCL group. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$) (B) The correlation between PPIA expression level and the inhibition of cell viability after NHL cell lines treated with 5 μg/ml CsA for 72 hours. Statistical significance was determined by Spearman test ($p = 0.0064$, $r2 = 0.6112$). There is a negative correlation between the relative PPIA expression and the%of viability after CsA treatment.

Supplementary



Supplementary figure 1. Representative 2 dimensional gel of LCL and NHL cell line. Silver stained 2 Dimensional SDS PAGE gels with the LCL cell line on the left and the SUDHL10 cell line on the right. PI ranges from 3 to 10 and molecular weight from 10 to 200 kDa. 1366 spots were found in the LCL cell line and 1300 in SUDHL10.

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Chapter 3

MYC expression and translocation analyses in low-grade and transformed follicular lymphoma

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Abstract

Aims Low-grade follicular lymphoma (FL) (grade 1/2, FL1/2) has an annual risk of transformation of ≈3%, which is associated with aberrations in *CDKN2A*/*B*, *TP53*, and *MYC*. As in diffuse large B-cell lymphoma, high MYC expression in transformed FL (tFL) might predict a *MYC* breakpoint.
Methods and results We quantified MYC expression by immunohistochemistry and digital analysis in 41 paired biopsies from 20 patients with FL1/2 with subsequent transformation and in four isolated biopsies of tFL. As controls, 28 biopsies of FL1/2 without transformation (median follow-up of 105 months) and nine biopsies of FL3A/B were analysed. In the 20 FL1/2–tFL pairs, MYC expression was significantly higher in tFL than in the initial FL1/2 biopsies (median 54% versus 6%; 7% in FL3A, and 35% in FL3B). *MYC* breaks (*MYC*-R) were detected in eight of 21 (38%) tFLs analysed by fluorescence *in-situ* hybridization (FISH), with a median MYC score of 86%. In two of the analysed tFL cases, the translocation was already detected in antecedent FL1/2. *MYC* partners were immunoglobulin (IG) loci in three of eight cases (one IGL, one IGH, and one IGK) and non-IG in five of eight cases (two *PAX5*, one *BCL6*, and two unknown). Of the eight *MYC*-R+ cases, six were *BCL2*+/*MYC*+ double-hit, one was *BCL2*+/*BCL6*+/*MYC*+ triple-hit, and one was *MYC*+ single-hit. All three *IG-MYC*+ cases showed a MYC expression level of >85%, whereas the five cases with a non-IG *MYC* partner had a wider range of expression (median 68%, range 13–86%). Among the 13 *MYC*-R− tFLs, two groups with almost dichotomous MYC expression could be observed (three cases showed ≥90% MYC expression), suggesting alternative mechanisms of MYC activation.
Conclusions We show an increase in MYC expression from FL1/2 to tFL. *MYC* breakpoints were present in ≈40% of the cases, which is markedly higher than in *de novo* DLBCL. MYC expression was uniformly high in cases with an IG-*MYC* translocation but much more heterogeneous and in part independent of the presence of a *MYC* break in non-IG-*MYC* and *MYC*-negative cases.

Introduction

Low-grade follicular lymphoma (FL) (grade 1/2, FL1/2) is an indolent B-cell lymphoma characterized by an annual risk of high-grade transformation (HT) of ≈3%, which is associated with an inferior prognosis.[1,2] It includes transformation into various histologies, most commonly diffuse large B-cell lymphoma (DLBCL), high-grade B-cell lymphoma (HGCBL) with *MYC* and *BCL2* and/or *BCL6* rearrangements, blastoid lymphomas [terminal deoxynucleotidyl transferase (TdT)-negative], and, very rarely, histiocytic sarcoma and TdT+ precursor B-cell lymphoma.[3-9] Recent studies have identified, among other mechanisms, mutations and deletions in *CDKN2A*/*B* and *TP53* as being involved.[5,10] *MYC* breaks have been detected in 25–50% of transformed FLs (tFLs), [4,5,10,11] and are usually acquired during the process of transformation. However, in rare cases, *MYC* breaks can be detected (already) in morphological FL1/2 lymphomas.[12-14] Several RNA expression studies have identified MYC up-regulation as an important pathway in transformation of FL1/2.[15-18] However, the dynamics of transformation in terms of MYC expression and how this correlates with the presence of *MYC* breaks and *MYC* translocation partners are unknown. We investigated and quantified MYC expression in a series of FL1/2 with and without subsequent transformation. In cases of transformation, both the FL1/2 and tFL samples were studied. Using a broad set of fluorescence *in-situ* hybridization (FISH) assays, we subsequently analysed the same samples for the presence of *MYC* breaks and tried to identify the *MYC* translocation partner.

Material and methods

Case Selection

All patients were selected from the files of the departments of Haematology and Pathology of the University Medical Centre Groningen, The Netherlands, and were diagnosed between 1986 and 2012. These included both patients admitted directly to the centre and patients with tFL secondarily referred from affiliated hospitals. For the present study, five different cohorts were defined: FL1/2 without subsequent transformation, FL1/2 with subsequent transformation, tFL, and, for comparison of the spectrum of MYC expression in FL, a separate series of *de-novo* FL3A and FL3B. The initial database search was conducted by application of the following selection criteria. For the FL1/2 group without subsequent transformation, a minimal follow-up of 50 months was required. For patients with tFL, a previously histologically confirmed (and centrally revised) FL1/2 was required (see below). By the use of a hospital-based haematology database and the nationwide histopathology and cytopathology data network and archive, tissue biopsies were traced and requested.[19] Case selection, revision and classification were performed according to the 2008 and 2016 World Health Organization classification by an expert haematopathologist

(P.M.K.).[20] For low-grade FL, only FL1/2 cases were accepted, and all cases with a distinct component of FL3A/B or transformation were excluded. Transformation was morphologically defined by a change into DLBCL, HGBCL, or TdT+ lymphoblastic lymphoma. FL3A/B was excluded. In cases of composite morphology in tFL samples, the high-grade component needed to be dominant and amenable to dissection/separate analysis. If multiple FL1/2 and/or tFL samples were available, the first biopsies were selected. If an initial sample was unavailable, the next biopsy in line with the same morphology was analysed. Only excision or core biopsies were used, and bone marrow and skin biopsies were excluded. The use of rest material is regulated under the code for good clinical practice in The Netherlands. Informed consent was not required, in accordance with Dutch regulations.

Immunohistochemistry

Freshly cut 3-µm-thick whole tissue sections were stained with an anti-cMYC rabbit monoclonal antibody (clone Y69; Ventana-Roche, Basel, Switzerland) on an automated immunostainer (Ventana-Roche), according to a routine protocol. Antigen retrieval was performed at 95°C for 64 min in a Tris-based CC1 buffer, pH 8.5 (Ventana-Roche) in a ready-to use-dilution.

Assessment of MYC Expression

MYC IHC-stained slides were scanned on a digital slide scanner (NanoZoomer 2.0; Hamamatsu, Hamamatsu city, Japan) and subsequently analysed with NDP VIEW software (Hamamatsu). First, the neoplastic follicles (in cases of FL) or diffuse areas with the highest MYC expression were selected by two investigators (R.v.P. and P.K.). These areas were further used for quantitative evaluation of MYC expression. Within these selected areas, five squares (0.16 mm^2 per square, five squares equivalent to 3.3 high-power fields when viewed directly by use of a standard microscope) were selected, and a virtual grid containing 154 intersections was projected over these squares. Each intersection was analysed for the presence of a MYC+ or a MYC− nucleus (Figure S1). The intensity of staining was not taken into account. The average percentage of MYC+ cells was calculated, and a standard error (SE) of the weighted proportion was calculated on the basis of the five selected squares. If the SE exceeded 2%, more squares were included in the counting until an SE of 2% had been reached. For validation of MYC IHC, staining of four benign paediatric hyperplastic tonsils and of three immunoglobulin (IG)–*MYC*+ Burkitt lymphomas was performed. To enable a more quantitative analysis and comparison between groups, MYC expression was analysed primarily as a continuous variable (in percentages). In addition, an arbitrary cut-off of ≥40% for MYC positivity was used, as is commonly used for DLBCL.[21]

FISH and Conventional Cytogenetics

Interphase FISH was performed on 3-µm-thick whole tissue paraffin sections as described in Supporting information Figure S2. All tFLs and the corresponding FL1/2 biopsies as well as the

separate FL3A/B biopsies were hybridized with both *MYC* break-apart (BAP) and IGH–*MYC* probes (Abbott-Vysis, Downers Grove, IL, USA), as hybridization with the BAP probe alone could miss rare IGH–*MYC*+ cases, e.g. insertions.[22, 23] All cases with a *MYC* break [referred to as *MYC* rearranged (*MYC*-R)+] and the corresponding FL1/2 biopsies (if available) were also analysed for *BCL2* and *BCL6* breaks with break-apart probe assays (Abbott-Vysis). To further determine the *MYC* translocation partner, all *MYC*-R+ but IGH–*MYC*– cases were analysed with the following homebrew dual-colour dual-fusion probe sets: IGK–*MYC*, IGL–*MYC*, 9p13/*PAX5*–*MYC*, and *BCL6*/*MYC* (Supporting information Figure S2). For the applied *MYC* FISH algorithm, see Figure S2. Conventional cytogenetic analysis was performed as previously described.[11]

Statistical Analysis

For comparison of MYC expression in paired and unpaired samples, the Wilcoxon signed rank test (matched pairs) and Mann–Whitney test, respectively, were used. Statistical analyses were conducted with GRAPHPAD PRISM, version 7.0 (GraphPad Software, La Jolla, CA, USA) and SPSS statistical software, version 20.0 (IBM SPSS Statistics for Windows; IBM Corp., NY, USA).

Results

Case Selection

In total, 82 biopsies were analysed, including 28 cases of FL1/2 without later histological transformation (median follow-up of 105 months, range 57–329 months), 20 pairs of FL1/2 with a later tFL (corresponding to 41 biopsies, as two transformed biopsies from one patient were analysed), four cases of tFL without initial FL1/2 biopsies available for further IHC and FISH analyses (for all cases, median time to transformation of 36 months, range 1–318 months), six cases of FL3A, and three cases of FL3B. The clinical characteristics at presentation of FL1/2 and treatment regimens are shown in Table 1.

MYC Expression in Different Grades of FL

As in the control tissues, an exclusively nuclear staining pattern was observed in all cases (representative images are shown in Figure 1A–D). In 67 of 82 biopsies evaluated (82%), counting of MYC+ nuclei in five areas was sufficient to reach an SE of ≤2%; in the other 15 cases (18%), up to 10 areas were necessary to reach this SE. The results for control tissues were according to the literature,[24] with MYC positivity rates of 11% in the germinal centres of four hyperplastic tonsils and of 98% in three Burkitt lymphomas (Figure 1A,B).

The median MYC IHC scores were comparable and not significantly different for FL1/2 without and with later transformation, i.e. 4% and 6%, respectively (Table 2), and were 7% for FL3A, 35% for FL3B, and 54% for tFL (Figure 2A). The MYC score was significantly higher in tFLs than in the

corresponding FL1/2 samples ($P < 0.001$; Figure 2B). With a cut-off of ≥40%, none of 28 FL1/2 cases without HT, one of 20 FL1/2 cases with HT, none of six FL3A cases, one of three FL3B cases and 15 of 24 tFL cases were MYC+ by IHC.

FISH

Seventeen of the 20 tFL biopsies of the FL1/2–tFL pairs, all four isolated tFL cases and all nine FL3A/B cases with material available for FISH analyses were successfully hybridized with both *MYC* BAP and IGH–*MYC* probes. In three tFL cases, no material for FISH was available. In the FL3A and FL3B samples, no *MYC* breaks were detected (0/6 and 0/3, respectively). Eight of the 21 analysed tFL cases (38%) showed a *MYC* break (*MYC*-R+; Table 3). In one case, the first tFL sample did not show a *MYC* break, but a biopsy taken 30 months later was *MYC*-R+ (both DLBCL morphology; case no. 1 in Figure 3). In one other case, we identified an unusual low-distance (maximal one to two signal diameters) separation between the centromeric and telomeric *MYC* signals in the *MYC* BAP assay, probably indicative of an insertion or inversion affecting the *MYC* locus, but we could identify neither any IG (IGH, IGK, or IGL) nor any non-IG (*BCL6*, 9p13/*PAX5*) insertion with the applied FISH assays. Although we cannot fully rule out another change affecting the *MYC* locus in this case, it was conservatively scored as *MYC* FISH-negative (Figure 2C). In six of eight cases, the involved *MYC* translocation partners could be identified with the applied FISH assays, and these included an IG partner in three cases (Table 3; Figures 3 and 4): the IG partners were IGL, IGK and IGH in one case each. Among the five cases without an IG partner (non-IG–*MYC*), the involved non-IG partners were 9p13/*PAX5* and *BCL6* in two cases and one case respectively. In the two remaining non-IG–*MYC* cases, the translocation partner could not be identified (negative with all applied FISH assays); these cases were assigned as 'non-IG-other'. Of the eight *MYC*-R+ tFL cases, six represented a *BCL2*+/*MYC*+ double-hit lymphoma, one was a *BCL2*+/*BCL6*+/*MYC*+ triple-hit lymphoma, and one was a *MYC*+ single-hit lymphoma; in the last of these, the corresponding FL1/2 also lacked a *BCL2* or *BCL6* translocation (case 1 in Figure 3 and Table 2; Figure S3). Of the six corresponding FL1/2 cases that could be investigated by FISH, one showed the *MYC* break (*PAX5*–*MYC*) already in a relapse FL1/2 sample taken 18 months after the initial FL1/2 diagnosis and 27 months before transformation (Table 2; Figure 3; Figure S4). In one other case that also had a 9p13/*PAX5*–*MYC* translocation in the tFL sample, t(8;9)(q24;p13)/9p13/*PAX5*–*MYC* was identified by conventional cytogenetics and FISH in a relapse FL1/2 sample taken 15 months after diagnosis and 7 months before transformation (Table 3; Figure 3; Figure S4). It is of note that, in both cases, the very first FL1/2 biopsy could not be analysed by FISH, so it remains unclear whether the *MYC* break was already present there.

Correlation Between MYC Expression and MYC Break in tFL

Seven of the eight *MYC*-R+ tFL cases showed MYC expression of ≥40%, whereas this level was attained in only six of 14 *MYC*-R–tFL cases (Table 2; Figures 2C, D, 3 and 4). Median MYC

expression values for the groups according to *MYC* break status (IG, non-IG, and *MYC*-R–) were 90%, 68%, and 29%, respectively, all three cases with an IG–*MYC* break having expression levels of >85%. Median MYC expression was higher but not statistically significant different between cases with and without a *MYC* break, but cases with an IG–*MYC* translocation had significantly higher MYC expression than those with a non-IG–*MYC* translocation ($P < 0.05$; Figure 2D). Interestingly, among the *MYC*-R–cases, two groups could be observed: one with high MYC expression of ≥40%, comparable to cases with a *MYC* break, and including three cases with expression levels of ≥90% (one with a possible insertion in the *MYC* locus with MYC expression of 95%); and a second with lower MYC expression (Figure 2C).

Clinical Correlates

The time line in Figure 3 summarizes data on MYC expression, *MYC* break status and clinical course in 24 individual tFL patients, in 20 of whom the (initial) FL1/2 biopsies were also investigated by FISH. The clinical course was very variable, with a median overall survival (OS) of 65 months (range 2–359 months), a median time-to-transformation (TTT) of 36 months (range 1–318 months), and a median OS after transformation of 20 months (range 0–108 months). No significant differences in outcome parameters were seen with respect to *MYC* status and MYC expression level (cut-off of ≥40%) at the time of transformation (data not shown). Table S1 summarizes the clinical characteristics of lymphomas at the time of transformation according to *MYC* status. The small number of patients and the retrospective nature of this cohort, patients with early transformation probably being selectively referred to our hospital, precludes the drawing of any conclusion about MYC expression in initial biopsies and clinical behaviour.

Discussion

In this study on the role of MYC expression and *MYC* breakpoints in FL and its transformation, we found higher MYC expression in FL3B than in FL3A and FL1/2, and even higher expression in tFL. Paired cases of FL1/2 and tFL showed a significant increase in expression with transformation. It is of note that two cases showed (slightly) lower MYC expression in the tFL sample, suggesting that other, MYC-independent, mechanisms are involved in transformation. The incidence of *MYC* breaks in the present study (38%) in tFL is markedly higher than the 5–15% reported in DLBCL.[25] This supports previous observations that, in spite of many similarities between tFL and *de-novo* DLBCL, genetic differences between these lymphoma subsets are also present.[5,26]

Similarly to what has been found in DLBCL [21,27] MYC expression levels were (although not statistically significantly) higher in tFL cases with a *MYC* break, and, as in many studies on DLBCL, a threshold of 40% staining cells could be used to select cases that should be analysed by FISH for a *MYC* break: only one of the eight cases with a *MYC* break had a MYC score of <40%.[28] Also, all *MYC*-R– FL3A/B cases had a MYC score of <40%. An unexpected finding was that high MYC expression levels were also found in cases without a *MYC* break. In particular, three tFL cases without a detectable *MYC* break (one of them with a potential small insertion or inversion within the *MYC* locus) had MYC expression levels of ≥90%, which is the level to be expected in Burkitt lymphoma. Therefore, other (not identifiable by FISH) structural or non-structural mechanisms, such as activating or stabilizing *MYC* mutations or overexpression related to differentially expressed microRNAs, could be responsible for the high MYC expression in these cases. Importantly, and in line with previous RNA expression studies that showed a role for alternative pathways in the transformation of FL, a significant proportion (9/24) of tFL cases showed low MYC expression of <40%.[15-17]

Using a broad panel of FISH assays, we were able to identify the *MYC* translocation partner in six of eight cases. The three IG–*MYC*– cases showed uniformly high (>85%) expression, and this level was significantly higher than that in non-IG–*MYC* cases. This relationship between the nature of the *MYC* partner and the level of MYC expression is also supported by a fourth tFL case with an IGL–*MYC* fusion and a MYC expression level of 97% that had to be excluded from the present series because it already showed focal transformation in the initial FL1/2 sample (Figure S5; Table S2). In non-IG–*MYC*-R cases, the expression levels were much more diverse. These data are in line with studies on DLBCL, and suggest that IG enhancers are more powerful in up-regulation of MYC than those of other genes.[22,29,30]

An interesting observation in our series is the frequent involvement of non-IGH partners and, in the case of an IG–*MYC* break, the frequent involvement of a light chain locus. Although the small number of cases precludes any statistical analysis, our findings are in line with observations in other *BCL2*+/*MYC*+ double-hit lymphomas, whereby non-IG locus involvement was found in ≈15–40% of cases, and light chain instead of heavy chain involvement was detected in 40–100% of cases.[31-35] This is in contrast to Burkitt lymphoma, which, by definition, has an IG–*MYC* single-hit configuration, and which shows variant IG light chain involvement in only 10–20% of cases.[36] One explanation may be that, in contrast to the translocation in Burkitt lymphoma and single-hit *MYC*-R+ cases of DLBCL, one IGH allele is already affected by t(14;18)(q32;21); in consequence, involvement of the other allele by another translocation may be deleterious for cases in which proper B-cell receptor signalling is necessary for tumour cell survival.[3] Indeed, these observations support the idea that these *MYC* breaks represent late(r) events, secondary to the initial t(14;18) translocation.[36,37]

We investigated whether the presence of a *MYC* breakpoint and/or overexpression of MYC might be associated with the behaviour of the tumour and patient outcome. No obvious association was found, the timing of a *MYC* break being randomly distributed, in some patients occurring >7 years after FL1/2 presentation. It is of note that rare patients with FL1/2 may show a *MYC* breakpoint,[12-14] as was observed in two of seven *MYC*+ tFL cases in which a (relapsed) antecedent FL1/2 biopsy could be analysed by FISH. In view of the small size of the series, its retrospective nature, with a possible referral bias, and the heterogeneity of the applied treatment regimens (including limited administration of anti-CD20-containing therapies), we are not able to draw any conclusions on the possible impact of MYC expression on clinical behaviour.

Conclusion

In conclusion, we showed an increase in MYC expression from FL1/2 to tFL. *MYC* breakpoints were present in ≈40% of the cases, which is a markedly higher proportion than in *de-novo* DLBCL. MYC expression was uniformly high in cases with an IG–*MYC* translocation, but much more heterogeneous and, in part, independent of the presence of a *MYC* break in non-IG–*MYC* and *MYC*− cases.

Tables

Table 1. Clinical characteristics and treatment regimens in 52 follicular lymphoma patients, specified according to the pre-transformation and post-transformation periods (if applicable)

	Without transformation (n = 28)	**With subsequent transformation (n = 24)**
Characteristics		
Male (%)	15 (53)	11 (46)
Median age (range)	55 (30-88)	51 (26-72)
Age		
< 60 year (%)	20 (71)	18 (75)
≥ 60 year (%)	8 (29)	6 (25)
Ann Arbor stage*		
I/II (%)	10 (36)	6 (25)
III/IV (%)	18 (64)	16 (67)
Unknown	0 (0)	2 (8)
Therapy pre-transformation period		
Rituximab		
Yes (%)	18 (64)	7 (29)
Chemotherapy		
Anthracyclines (%)	6 (21)	7 (29)
Purine analoge (%)	4 (14)	1 (4)
Alkylating agents (%)	19 (68)	15 (63)
AutoSCT (%)	1 (4)	1 (4)
Radiotherapy (%)	10 (36)	10 (42)
Therapy post-transformation period		
Rituximab		
Yes (%)	N.A.	15 (63%)
Chemotherapy		
Anthracyclines (%)	N.A.	19 (79%)
Purine analoge (%)	N.A.	0
Alkylating agents (%)	N.A.	23 (96)
AutoSCT (%)	N.A.	8 (33)
Allo-SCT	N.A.	2 (8)
Radiotherapy (%)	N.A.	7 (29)

Abbreviations: N.A, not applicable

Table 2: MYC immunohistochemistry results in 48 FL1/2 samples at diagnosis and in 21 tFL

Pathology	FL without transformation (n = 28)	FL with subsequent transformation (n = 20)*	tFL MYC-FISH negative (n=14)*	tFL MYC-FISH positive (n=8)
Median % (range)	4 (1-27)	6 (1-42)	27 (1-95)	86 (13-94)
< 40% (%)	28 (100)	19 (95)	8 (57)	1 (13)
> 40% (%)	0 (0)	1 (5)	6 (43)	7 (87)

*Of the 52 follicular lymphoma patients samples at diagnosis four were not available for this analysis. Of the 24 patients with subsequent transformation MYC-FISH status was available for 22 samples of 21 patients. *Note that case Nr. 4 from Table 3 and Figure 5 is included twice in the analysis: both in the MYC-R negative group (first tFL sample MYC-R negative) and in MYC-R+ group (2nd tFL sample MYC-R+).*

Table 3: Histopathologic and genetic characteristics of *MYC*-R-positive tFL samples and their corresponding FL1/2 samples

Case	Age	TTT (M)	Sex	Histology	CD10	BCL2	Ki67 % #	*MYC* BAP	IGH-*MYC*	*MYC*-partner	MYC IHC score %	*BCL2* BAP	*BCL6* BAP	Karyotype
1-FL1/2	50	56	M	FL1/2	Pos	Pos	36	Neg	Neg		2	Pos	Neg	
1-tFL‡	57			DLBCL	Pos	Pos	NA	Pos	Neg	Non-*IG* unknown	86	Pos	Neg	
2-FL1/2	45	318	F	FL1/2	Pos	NA	Low	Neg	Neg		5	Neg	Neg	
2-tFL	72			HGBCL, NOS	Pos	Pos	83	Pos	Pos	IGH	88	Neg	Neg	48,XX,+X,t(8;14)(q24;q32),dup(12)(q11q15),del(15)(q11q21)+22[8]/46,XX[2] §
3-FL1/2	55	32	M	FL1/2	Pos	NA	NA	Neg	Neg		7	Pos	Neg	
3-tFL	57			B-LBL (TdT+)	Pos	Pos	89	Pos	Neg	IGL	90	Pos	Neg	
4-FL1/2	71	19	M	FL1/2	Pos	Neg	46	Neg	Neg		11	Pos	Neg	
4-tFL	72			DLBCL	Pos	Pos/ Neg	52	Pos	Neg	*BCL6*	54	Pos	Pos	
5-FL1/2	54	8	F	FL1/2	Pos	Pos	37	Neg	Neg		17	Pos	Neg	
5-tFL	55			MYC+ HGBCL	Pos	Pos	81	Pos	Neg	Non-*IG* unknown	68	Pos	Neg	
6-FL1/2*	68	45	M	FL1/2	Pos	Pos	21	Pos	NA	9p13/ *PAX5*	22	Pos	Neg	43~47,add(9)(p1),add(10p),del(18)(q2),+21,+mar[4]
6-tFL	70			DLBCL/FL	Pos	Pos	51	Pos	Neg	9p13/ *PAX5*	13	Pos	Neg	48,XY,+3,–4,add(8)(q2?4),add(9)(p11),del(13)(q14~22q22~32),t(14;18)(q32;q21),+21,+mar[7]/46,XY[8]
7-tFL†	52	22	M	DLBCL	NA	NA	61	Pos	Neg	9p13/ *PAX5*	86	Pos	Neg	
8-tFL	61	131	M	DLBCL	Neg	Pos	26	Pos	Neg	IGK	94	Pos	Neg	

Case numbers in Table also refer to individual cases in Fig. 4.
HGBCL: high grade B-cell lymphoma; NOS, not otherwise specified ; LBL, lymphoblastic lymphoma ; NA, not available ; NOS, not otherwise specified ; TTT, time-to-transformation
 # except for case 1 FL1/2 Ki67 was counted in at least 200 cells.
**represent FL1/2 relapse, original FL1/2 biopsy (age 66) not available for analysis.*
†previous FL1/2 biopsy not available for analysis by IHC but conventional cytogenetics showed t(8;9)(q24;p13) as subclonal aberration. MYC-9p/PAX5 was confirmed by FISH
48,XY,der(3)t(3;15)?(p21;q15~21),+7,t(8;9)(q24;p13),del(11)(q23),?dup(12)(q24.3q15),t(14;18)(q32;q21),t(14;22)(q32;q11.2),del(15)(q15),der(19)t(3;19)(p21;p13.3),+mar1[14]/48,XY,add(2)(q33),–5,+7,del(11)(q23),?dup(12)(q24.3q15),t(14;18)(q32;q21),t(14;22)(q32;q11.2),add(16)(p11.2),+mar1,+mar2[3]/46,XY [5]
‡sample is second transformed lymphoma biopsy. First transformed biopsy was MYC break negative.
§Conventional cytogenetics of previous FL1/2 sample 4 years earlier (not analyzed by MYC IHC and FISH): 46,XX,dup(12)(q11q15)[6]/46,XX[4]

Figures

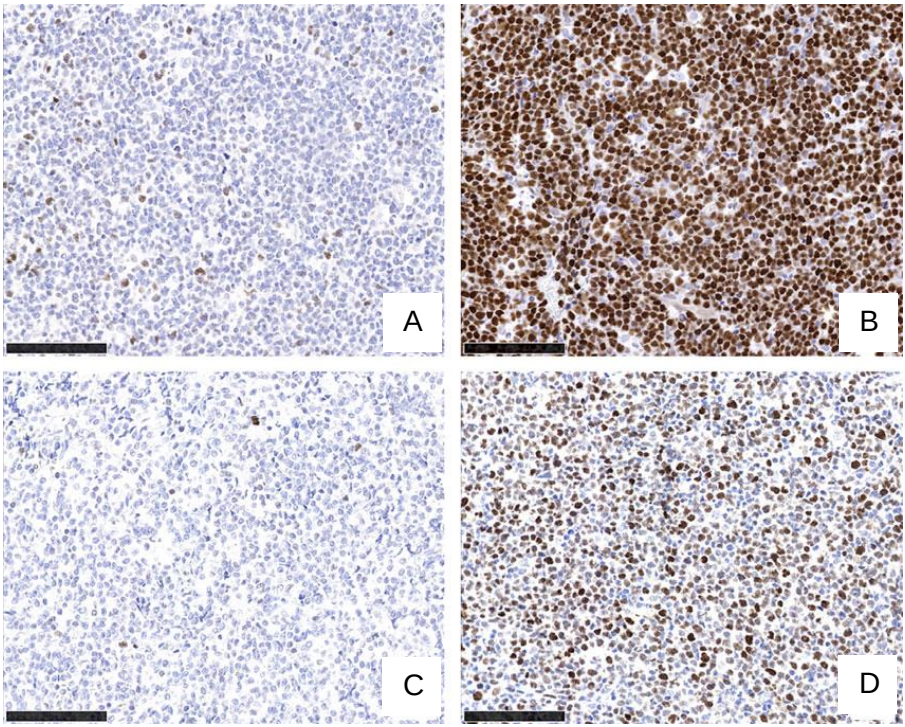


Figure 1. Representative images of MYC immunohistochemical staining (% of positive staining refers to the individual area shown in the figure, and not necessarily to the average value over multiple areas). A,B, Benign tonsil (7% positive) (A) and IGH–MYC+ Burkitt lymphoma (98% positive) (B). C,D, Follicular lymphoma (FL)1/2–transformed FL (tFL) diagnosis transformation pair with (C) MYC-R□ FL1/2 (2%) and (D) tFL non-IG–MYC (86%). IG, immunoglobulin.

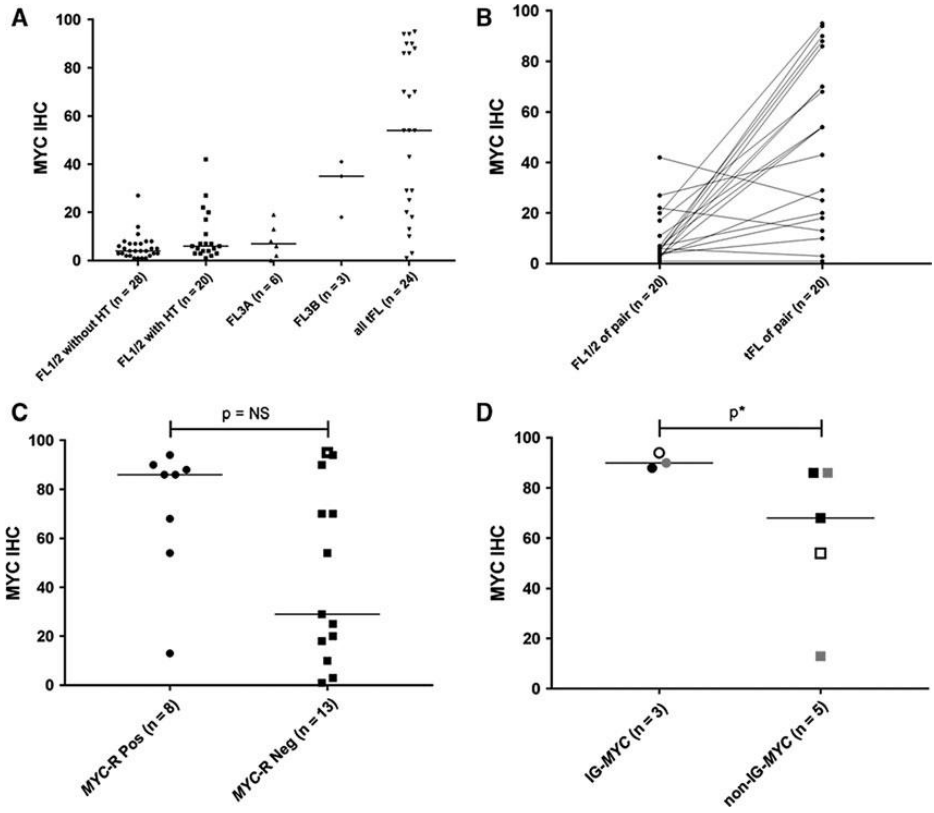


Figure 2: A, Overview of MYC immunohistochemistry (IHC) scores in different follicular lymphoma (FL) cohorts. B, Dynamics of MYC expression in individual cases of FL1/2 and HT FL patient pairs with MYC IHC for both time points available. MYC in transformed FL (tFL) versus FL1/2 ($P < 0.001$). C, Assessment of MYC IHC scores in HT FL according to *MYC* break status (cases with both IHC and *MYC* fluorescence *in-situ* hybridization available only). □, case with possible *MYC* insertion. D, Assessment of MYC IHC scores in HT FL according to *MYC* translocation partner. ○, IGK–*MYC*; ●, IGL–*MYC*; •, IGH–*MYC*; ■, unknown–*MYC*; ■, *PAX5*–*MYC*; □, *BCL6*/*MYC*. *$P < 0.05$. HT, high-grade transformation; IG, immunoglobulin.

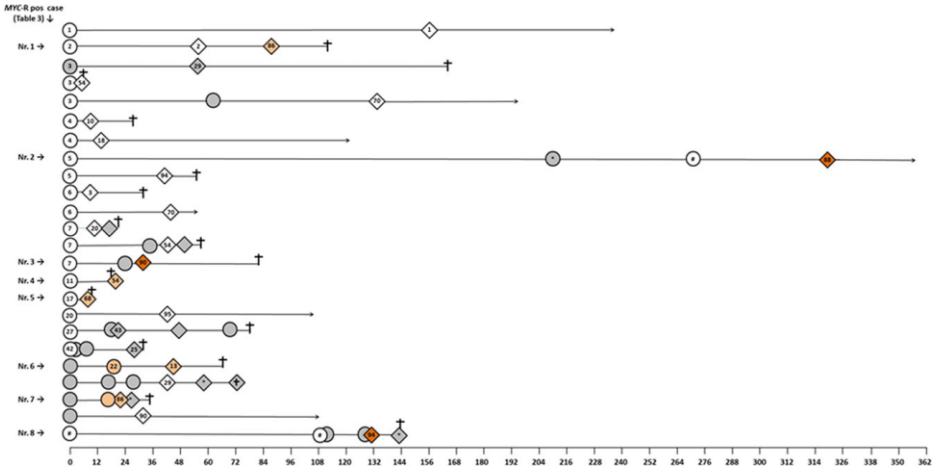


Figure 3. Timeline showing MYC expression, *MYC* break status and clinical course in all 24 individual patients. Case numbers of *MYC*-R+ cases in this figure also refer to individual cases in Table 3; cases without a number represent *MYC*-R− cases. Numbers in circles and squares indicate percentages of MYC positivity (MYC immunohistochemistry score). ○, FL1/2, *MYC*−; , follicular lymphoma (FL)1/2, non-IG–*MYC*+; , FL1/2, *MYC* status not available; ◇, transformed FL (tFL), *MYC*−; , tFL, non-IG–*MYC*+; , tFL, IG–*MYC*+; , tFL, *MYC* status not available; , tFL, extensive disease at autopsy; †diseased; *cytology sample; #*MYC*− by conventional cytogenetics; →, length of follow-up. For readability, metachronous/synchronous bone marrow staging and follow-up biopsies are not displayed in the figure. Time-to-transformation was calculated from the first histological samples. Cytological punctures are not displayed in the figure (unless stated otherwise, e.g. when no metachronous/synchronous histological biopsy was available). Cytogenetics from other haematological malignancies, e.g. secondary acute myeloid leukaemia after stem cell transplantation, are also omitted from the figure. Cases are ordered according to the MYC score in the FL1/2 biopsy (if available). IG, immunoglobulin

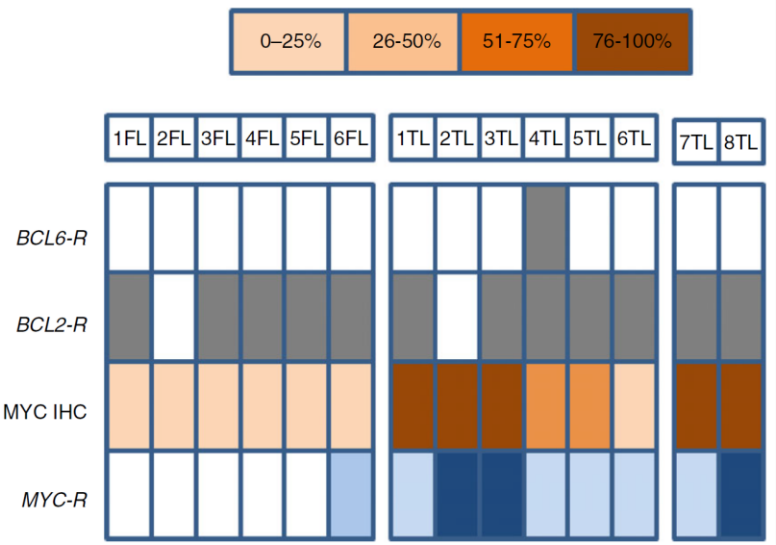


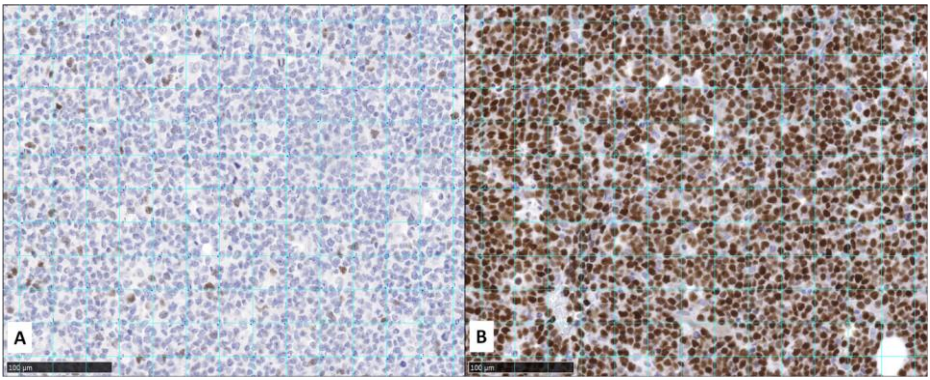
Figure 4. Heatmap of MYC-R+ transformed follicular lymphoma (tFL) cases and corresponding follicular lymphoma (FL)1/2 counterparts analysed by immunohistochemistry (IHC) and fluorescence in-situ hybridization (if available). Grey indicates the presence of bcl-2 or bcl-6 breaks, and empty boxes indicate the absence of these. Light blue indicates non-IG–MYC translocation, and dark blue indicates IG–MYC. The colour key (top) refers to the MYC IHC score (in quartiles). Numbers in headers refer to individual cases in Table 3 and Figure 4, with FL referring to the FL1/2 sample and TL to the tFL sample. IG, immunoglobulin

Supplementary

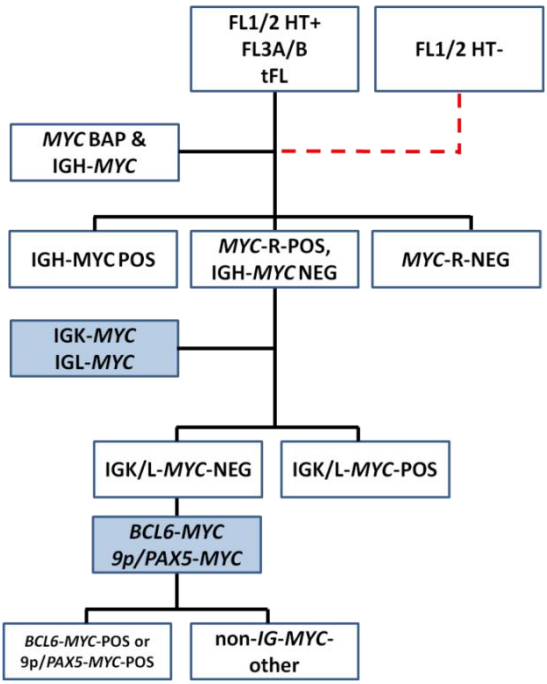
Supplemental table 1: Clinical characteristics of 21 transformed follicular lymphoma patients at time of transformation available for *MYC*-FISH interpretation.

Characteristics	*MYC*-R negative (N=14)[∫]	*MYC*-R positive (N= 7)[∫]
Male	5 (36%)	5 (71%)
Median age (range)	54 (37-72)	61 (51-72)
Median Time to transformation in months (range)	35 (1-156)	39 (9-318)
Stage *		
I/II (%)	2 (14%)	3 (43%)
III/IV (%)	11 (79%)	4 (57%)
Unknown	1 (7%)	0 (0%)
IPI-score		
Low risk (0-1)	3 (21%)	3 (43%)
Intermediate (2-3)	5 (36%)	2 (29%)
High risk (4-5)	6 (43%)	2 (29%)

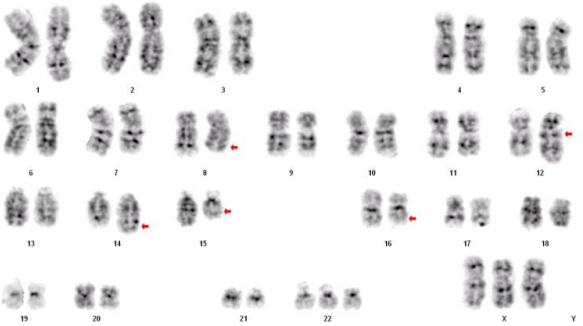
** 1 missing*
[∫]Note that number of MYC-R negative and MYC-R positive is 14 and 7 respectively as clinical characteristics refer to MYC-status in first analyzed tFL biopsy and this case (nr. 1) being MYC-R negative in first but MYC-R positive in second analyzed tFL biopsy.



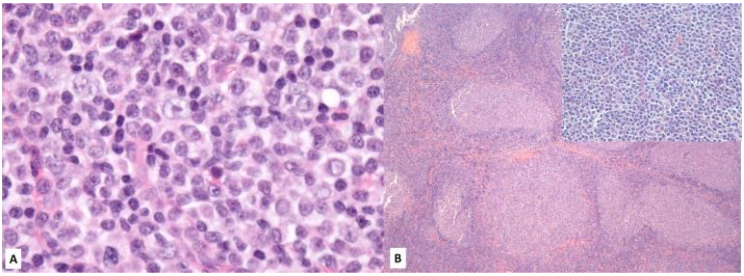
Supplementary figure 1: Assessment of MYC expression.



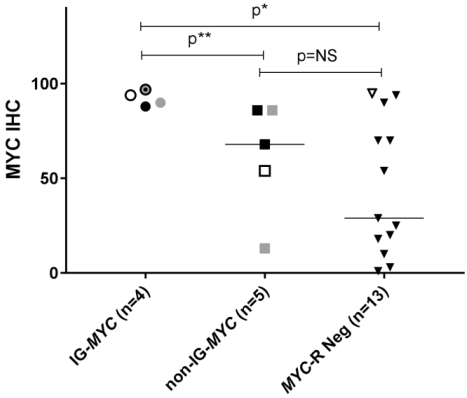
Supplementary figure 2: Applied *MYC* FISH algorithm.



Supplementary figure 3: Karyotype of case 1.



Supplementary figure 4: Low-grade morphology of FL1/2 with *MYC* break.



Supplementary figure 5: Assessment of MYC IHC scores in HT FL according to *MYC* status and translocation partner.

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Chapter 4

HLA dependent immune escape mechanisms in B-cell lymphomas: Implications for immune checkpoint inhibitor therapy?

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Abstract

Antigen presentation by tumor cells in the context of Human Leukocyte Antigen (HLA) is generally considered to be a prerequisite for effective immune checkpoint inhibitor therapy. We evaluated cell surface HLA class I, HLA class II and cytoplasmic HLA-DM staining by immunohistochemistry (IHC) in 389 classical Hodgkin lymphomas (cHL), 22 nodular lymphocyte predominant Hodgkin lymphomas (NLPHL), 137 diffuse large B-cell lymphomas (DLBCL), 39 primary central nervous system lymphomas (PCNSL) and 19 testicular lymphomas. We describe a novel mechanism of immune escape in which loss of HLA-DM expression results in aberrant membranous invariant chain peptide (CLIP) expression in HLA class II cell surface positive lymphoma cells, preventing presentation of antigenic peptides. In HLA class II positive cases, HLA-DM expression was lost in 49% of cHL, 0% of NLPHL, 14% of DLBCL, 3% of PCNSL and 0% of testicular lymphomas. Considering HLA class I, HLA class II and HLA-DM together, 88% of cHL, 10% of NLPHL, 62% of DLBCL, 77% of PCNSL and 87% of testicular lymphoma cases had abnormal HLA expression patterns. In conclusion, an HLA expression pattern incompatible with normal antigen presentation is common in cHL, DLBCL, PCNSL and testicular lymphoma. Retention of CLIP in HLA class II caused by loss of HLA-DM is a novel immune escape mechanism, especially prevalent in cHL. Aberrant HLA expression should be taken into account when evaluating efficacy of checkpoint inhibitors in B-cell lymphomas.

Introduction

In the past decade, cancer immunotherapy has made major advances by targeting a series of cell surface molecules known as immune checkpoints. The checkpoint molecules can repress the function of killer and pro-inflammatory lymphocytes. Checkpoint inhibitors are monoclonal antibodies (mAbs) that block these inhibitory receptors, thereby stimulating T-cells and generating an antitumor response.[1-3] B-cell lymphoma comprise a heterogeneous group of malignancies, which arise from malignant transformation of B-cells.

The mAbs against programmed death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) have shown substantial therapeutic activity in heavily treated classical Hodgkin lymphoma (cHL) and encouraging results in relapsed/refractory diffuse large B-cell lymphoma (DLBCL) with overall response rates (ORR) of 87% and 36% respectively.[4-8] Despite these encouraging results, complete remissions are rare and it remains to be established which patients benefit most from checkpoint inhibition.

Antigen presentation depends on the proper processing of proteins and presentation of peptides through the human leukocyte antigens (HLA).[9] Normal B-cells present antigens through HLA class I, like any other nucleated cell, and as professional antigen-processing cells also in the context of HLA class II.[10,11] In cancer cells, so called neo-antigens can arise from proteins that are altered, e.g. by gene mutations. Presentation of these neo-antigens by HLA should induce antitumor immune responses. However, lymphoma cells can prevent these responses by various immune evasive mechanisms, including expression of immune checkpoint molecules.[12,13] Another mechanism to prevent antitumor immune responses involves loss or aberrant expression of HLA, which precludes presentation of tumor cell specific antigens. Thus, loss or aberrant HLA expression may very well have an impact on the efficacy of checkpoint inhibitors.

Loss of membranous HLA class I and/or HLA class II expression has frequently been described in B-cell lymphomas, including cHL, DLBCL, primary mediastinal B-cell lymphoma (PMBCL) and the immune privileged aggressive B-cell lymphomas of brain and testis.[14-27] Observations in several B-cell non-Hodgkin lymphomas (NHL) indicate cytoplasmic retention of HLA-molecules in a proportion of patients.[17,20,25,27,28] In three cHL lymph node cell suspensions, it has been described that disruption of antigen presentation is caused by retention of the class II-associated invariant chain peptide (CLIP) in the membranous HLA class II molecules.[16] HLA-DM is essential in the intracellular assembly of HLA class II-antigenic peptide complexes. It displaces CLIP from the antigen binding groove of HLA class II molecules, to make this groove accessible for loading of antigens. Lack of HLA-DM results in an apparently normal cell surface expression of HLA class II, but without presentation of antigens and neo-antigens.[9]

In this study, we examined the combined protein expression patterns of HLA class I and HLA class II in a large set of cHL, nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), DLBCL, primary central nervous system lymphoma (PCNSL) and primary testicular lymphoma. We expanded this with HLA-DM as well as CLIP in a subset of patients to determine whether the lymphoma cells apply this alternative mechanism of inducing functional loss of HLA class II antigen presentation.

Material and methods

Patients and tumor samples

Primary diagnostic FFPE tissue blocks of 389 cHL, 22 NLPHL, 137 DLBCL not otherwise specified 39 PCNSL and 19 primary testicular lymphoma patients were retrieved from the tissue banks of the pathology department of the University Medical Center Groningen and affiliated hospitals between 1987 and 2011. Part of the HLA expression data in cHL (n = 292) was described previously in relation to clinical outcome.[15] In addition, frozen tissue sections from 20 cHL patients included in the cohort of 389 patients, were included. All cases were reviewed by two experienced hematopathologists. To determine the prognostic value of aberrant HLA expression in DLBCL clinical data on the patients was retrieved from the electronic hospital database of the University Medical Center Groningen. The primary clinical end point was PFS, defined as the time from treatment until relapse or death. Follow-up was completed until December 2015. Patients were treated according to best practice. IHC was performed on anonymized tissue sections in compliance with national ethical guidelines (“Code for Proper Secondary Use of Human Tissue,” Dutch Federation of Medical Scientific Societies) and the declaration of Helsinki.

Immunohistochemistry and scoring

Tissue sections of 3 µm were cut from formalin fixed and paraffin embedded tissue samples. Immunohistochemical staining was performed according to standard procedures. Briefly, sections were dewaxed with xylene and endogenous peroxidase was blocked. Antigen retrieval was performed in 10 mM Tris (tris-hydroxymethyl-aminomethane)/1 mM EDTA (ethylene diamine tetracetic acid) at pH 9.0. Staining was visualized with mouse mAbs HC10 (HLA class I heavy chains, 1:500, kindly provided by Prof. Dr J. Neefjes, the Netherlands Cancer Institute, Amsterdam), B2M (1:200, DAKO, Glostrup, Denmark), HLA DR/DQ/DP (HLA class II, 1:500, DAKO) and HLA-DM (1:200, BD Biosciences, Breda, the Netherlands). Primary antibodies were detected by secondary and tertiary conjugate antibodies. All cases were stained for B2M, HLA class I and HLA class II. HLA-DM staining was performed in all DLBCL and PCNSL cases and in the cHL cases with membranous HLA class II expression. CLIP staining was performed on fresh frozen material of 20 cHL patients with anti-CLIP (CerCLIP 1:200 BD) and HLA-DM staining with

anti HLA-DM (1:200 BD). For all stainings, normal tonsil tissue was used as a positive control. In the cHL cases, EBV status was determined by in situ hybridization using a probe specific for EBV encoded RNAs (EBERs). Scoring was performed by two experienced hematopathologists. Discrepant cases were subsequently discussed until consensus was reached. For HLA class I and HLA class II, membranous staining in the majority of tumor cells was considered normal. Per case, HC10 and B2M were scored consecutively and membranous staining was concordant for all cases. For HLA-DM cytoplasmic staining was considered as normal and for CLIP membranous staining was scored as abnormal.

Statistical analysis

All categorical variables were expressed as percentages. Where applicable, differences between groups were evaluated by chi-square (for binary variables). A two-tailed p value of less than 0.05 indicated statistical significance. PFS curves were estimated according to the Kaplan–Meier method. Between-group differences in PFS were evaluated using the log-rank test. All analyses were performed using IBM SPSS Statistics version 22.

Results

HLA expression in cHL

Of the 389 cHL samples, 28 were not evaluable for HLA class I and/or HLA class II and were excluded from further analysis. Normal HLA class I cell surface expression was observed in tumor cells in 132 out of 361 cases (36.6%), significantly more often in EBV+cHL (72.6%) than in EBV-cHL (16.8%) ($p < 0.001$). Various aberrant staining patterns were observed, including complete lack of both B2M and HC10 in 52.8% of negative cases (Fig. 1). Of the HLA class I negative cases 38.1% had only cytoplasmic B2M staining and in 8.3% only cytoplasmic HC10 staining. Cell surface expression of HLA class II was present in 214 out of 361 cHL cases (59.3%), significantly more often in EBV+cHL (70.3%) than in EBV-cHL (53.2%) ($p < 0.001$) (Table 1). In 40.1% of the HLA class II cell surface negative cases cytoplasmic staining was observed.

CLIP and HLA-DM expression in cHL

Twenty HLA class II membrane positive cases, for which frozen material was available, were stained for CLIP and HLA-DM. CLIP staining was clearly membranous in eight cases, indicating diminishment or lack of presentation of immunogenic peptides in the context of HLA class II. In all of these eight cases HLA-DM staining was completely negative, both in frozen and corresponding formalin-fixed paraffin-embedded (FFPE) tissue. In the 12 cases with normal CLIP staining (weak cytoplasmic), HLA-DM was expressed (Fig. 2). HLA-DM staining was then performed on an additional 69 HLA class II positive FFPE cases. In total, HLA-DM expression was lacking in 44 out of 89 cases of cHL (49.4%).

HLA expression in NLPHL

Of the 22 NLPHL cases, 1 was not evaluable for HLA class II and was excluded from further analysis. The majority of cases (90.5%) had normal HLA class I and HLA class II expression. Loss of HLA class II was observed in one case and combined loss of HLA class I and class II in another case. None of the HLA class II positive cases had HLA-DM loss.

HLA expression patterns in DLBCL

Of the 137 DLBCL samples, 20 were not evaluable for either HLA class I, HLA class II or HLA-DM and were excluded from further analysis. HLA class I surface expression was observed in 53 out of 117 cases (45.3%) (Table 2). Of the HLA class I membrane negative cases, two showed cytoplasmic HC10 staining. Cytoplasmic B2M was detected in 31 of the 64 (48.4%) HLA class I negative cases, these did not include the two HC10 cytoplasmic staining cases. Cell surface HLA class II expression was observed in 78 out of 117 DLBCL cases (66.7%). Of the negative membrane cases, cytoplasmic HLA class II expression was seen in 10 out of 39 (25.6%). We found that in the 78 HLA class II cell surface expressing tumor cells, HLA-DM expression was lacking in 11 cases (14.1%). Representative examples of the various staining patterns observed for HC10, B2M and HLA class II are shown in Fig. 2.

HLA-expression patterns in PCNSL and testicular lymphoma

Of the 39 PNCSL samples, 5 were not evaluable for either HLA class I, HLA class II or HLA-DM and were excluded from further analysis. HLA class I and HLA class II cell surface expression was observed in 29.4% of cases, whereas there was a loss of HLA class I and HLA class II in 24 out of 34 (70.6%) and 19 out of 34 (55.9%) cases, respectively (Table 2). HLA-DM loss was observed in 1 case (2.9%). Of the 19 primary testicular lymphoma, 4 were excluded because of missing staining data. Two samples (13.3%) showed normal HLA expression, while 12 samples (80%) lacked both HLA class I and HLA class II (Table 2)

Combined expression patterns in cHL, NLPHL, DLBCL, PCNSL and testicular lymphoma

Combined results of functional cell surface HLA class I and II expression are presented in Table 3. These combined analyses show that only 12.4% of cHL, 37.6% of DLBCL, 23.5% of PCNSL and 13.3% of testicular lymphoma show an HLA expression pattern that is compatible with normal antigen presentation. In contrast, 90.5% of NLPHL cases show normal HLA class I and HLA class II expression. In EBV-cHL the most prevalent pattern is the combined loss of HLA class I and HLA class II, whereas in EBV+cHL there is more often disruption of HLA class II signaling, either through HLA class II or HLA-DM loss (Table 1). In DLBCL the most frequent aberrant HLA expression is the loss of both HLA class I and HLA class II (35%). Isolated HLA class I loss (19.7%) is more common than isolated loss of HLA class II and HLA-DM (7.7% and 4.3%) (Tables 2 and 3). The HLA expression patterns were not related to gender, age or stage of the disease (Table S1). In the cohort of patients treated with R-CHOP, we observed no significant

difference in progression free survival (PFS) between patients with a normal HLA pattern or aberrant HLA pattern (Log Rank *p* value 0.25) (Fig. S1). PCNSL and testicular lymphoma have the highest frequency of combined HLA class I and HLA class II loss (52.9% and 80%), with isolated loss of HLA class I or HLA II only being observed incidentally. HLA-DM loss does not seem to constitute a recurrent mechanism for immune escape in either PCNSL or primary testicular lymphoma.

Discussion

As proper antigen presentation in the context of HLA is expected to be a prerequisite for the action of immune checkpoint inhibitors, we studied HLA expression in retrospective cohorts of B-cell lymphoma. We showed that only a minority of cHL, DLBCL, PNCSL and testicular lymphoma cases show HLA expression that is compatible with normal antigen presentation for both HLA class I and II. Combined loss of HLA class I and HLA class II was the most prevalent aberrant pattern in all these lymphoma. In cHL and DLBCL, we identified loss of HLA-DM as a novel mechanism causing disruption of normal antigen presentation in the context of HLA class II. The minimal requirement for B-cell lymphoma tumor cells to act like professional antigen presenting cells is cell surface expression of the HLA class I heavy chain-B2M complex and the HLA class II heavy chain dimer. Immunohistochemistry (IHC) for these components in normal germinal center B-cells shows strong membranous staining and weak cytoplasmic staining. In EBV-cHL, HLA class I expression has previously been reported to be lacking in 55% (*n* = 38), 81% (*n* = 21) and 71% (*n* = 14) of cases [29-31] while, we found 83.2% (*n* = 233). In EBV+cHL these percentages are much lower: 8% (*n* = 25), 24% (*n* = 17), 25% (*n* = 24) and in our series loss of HLA class I was observed in 27.4% (*n* = 128).

In previous publications on DLBCL a wide range of HLA class I loss has been reported, ranging from 34% to 75%,[19-22] compared with 54.7% in our study. The lower values in this range correspond to studies that made no distinction between membranous and cytoplasmic staining in IHC. Loss of HLA class II expression is consistently less frequently reported than HLA class I loss.[24-27] Importantly, in the majority of these studies no aberrant cytoplasmic HLA class II staining was described and probably included as positive. In a recent study, specifically looking at cytoplasmic HLA class II expression, aberrant expression patterns were observed in 41%. Interestingly, aberrant HLA class II expression was more often observed in the non-germinal center B-cell type.[28] Taking this into account, as well as differences in methodology and antibodies used, the results of these studies are compatible with our observed loss of membranous HLA class II expression in 33.3% of cases.

Our IHC results indicate that there is a variety of mechanisms involved in the lack of cell surface HLA expression, judging from different staining patterns in the cytoplasm (absent, diffuse, granular or with Golgi-like localization). In PCNSL and primary testicular lymphoma, loss of HLA-I and HLA-II is frequently due to homozygous or heterozygous deletions of the HLA loci on chromosome 6p21.[18,19] Both in cHL and DLBCL recent studies indicate that mutations of B2M are a common mechanism for HLA class I loss.[20,32,33] as B2M is required for the stabilization of the HLA class I heavy chain. Decreased HLA class II expression in DLBCL is believed to arise through repression of the HLA locus by decreased expression of CIITA.[27,34-38] The mechanism behind this repression is unclear. Although CIITA alterations are common in primary mediastinal B-cell lymphoma and result in loss of CIITA.[39] mutations of CIITA in DLBCL are infrequent (0–9%) and can only partially explain the loss of HLA class II.[32,40-43] In cHL, 15% of cases harbor a translocation of CIITA, resulting in an incomplete downregulation of HLA class II.[44] In addition, we previously found mutations in 2 out of 6 cHL cell lines.[45] Whatever the mechanism is, downregulation of HLA is probably a response to continuous antitumor immune responses that increase over time with emergence of antigenic peptides that are related to malignant transformation or disease progression.

Another immune evasive mechanism that we found to be frequent in cHL cases is retained localization of CLIP in the antigen binding groove of HLA class II. In a previous publication it was shown that in three fresh cHL affected lymph node cell suspensions cell surface HLA class II was not occupied by antigenic peptides, but by the non-immunogenic CLIP.[16] We have now shown that this is caused by lack of expression of HLA-DM. HLA-DO is another HLA accessory molecule that counteracts HLA-DM, but we found no increased expression of HLA-DO (results not shown). Interestingly, presentation of CLIP by dendritic cells antagonizes Th1 polarization. Thus, presentation of CLIP by Hodgkin tumor cells may contribute to the predominant Th2/Treg T-cell populations that are known to directly surround these tumor cells.[46]

Loss of HLA in B-cell lymphoma has been shown to be related to a decrease in number of tumor infiltrating lymphocytes and diminished interferon-gamma responses.[18,21] Both in cHL and DLBCL lack of membranous HLA class II expression on tumor cells has been shown to be an independent adverse prognostic factor.[15,25,26] However, with the introduction of rituximab the prognostic value of HLA class II in DLBCL has become less clear.[47] It was recently suggested that cytoplasmic HLA class II conveys a worse prognosis when compared with HLA class II membrane or HLA class II negative staining.[28] In our current cohort of DLBCL patients treated with R-CHOP, there was no significant difference in PFS between patients with normal HLA expression and patients with (combined) aberrant expression patterns.

Disrupted antigen presentation is expected to have important implications for the efficacy of checkpoint inhibitors. Despite a high ORR in relapsed cHL treated with a checkpoint inhibitor only 17% of patients achieve a complete remission.[5] In relapsed DLBCL, checkpoint inhibition as monotherapy shows modest efficacy (ORR of 36%).[6] However, checkpoint inhibition appears more effective when applied as a consolidation strategy (ORR 51%).[8] Lack of HLA class I results in loss of presentation of tumor derived neo-antigenic peptides and makes the tumor cells unrecognizable to $CD8^+$ cytotoxic T-cells. This implies that the rationale for using immune checkpoint inhibitors might be restricted to HLA class I positive cases. Loss of cell surface expression of HLA class II on the tumor cells may not be a problem, as priming of antitumor immune responses can also occur through professional antigen presenting cells present in the micro-environment. However, in melanoma response to a PD-1 inhibitor does depend on presence of HLA class II on the tumor cells.[48] Since, aberrant HLA expression is the most prevalent finding that potentially can hamper the efficacy of checkpoint inhibitors, future clinical trials with checkpoint inhibitors should consider HLA expression, both in lymphomas and other cancers.

Conclusion

In conclusion, the majority of cHL, DLBCL, PCNSL and primary testicular lymphoma show HLA expression that is incompatible with normal antigen presentation. The combined losses of HLA class I and HLA class II represent the most frequent mechanism of immune escape. Loss of HLA-DM resulting in the loss of antigen presentation through HLA class II presents a novel mechanism of immune escape in cHL and DLBCL. Our data implicate the importance of taking HLA expression into account when evaluating efficacy of checkpoint inhibitors.

Tables

Table 1. HLA class I, HLA class II and HLA-DM staining patterns in tumor cells of 361 classical Hodgkin lymphoma patients

HLA			Hodgkin			
I	**II**	**DM**	**Total %** (n = 361)	**EBV+ %** (n = 128)	**EBV- %** (n = 233)	**p-value**
+	+	+	12.4	27.7	4.1	< 0.01
+	-	ND	11.1	15.6	8.6	0.11
+	+	-	13.0	29.3	4.1	< 0.01
-	+	+	17.6	3.8	25.4	< 0.01
-	-	ND	29.7	14.1	38.2	< 0.01
-	+	-	16.2	9.5	19.6	0.03

Table 2: HLA class I, HLA class II and HLA-DM tumor cell staining patterns in 117 diffuse large B-cell lymphoma, 34 primary central nervous system lymphoma and 15 primary testicular lymphoma patients

HLA			**DLBCL %**	**PCNSL %**	**Testicular %**
I	**II**	**DM**	(n = 117)	(n = 34)	(n = 15)
+	+	+	37.6	23.5	13.3
+	-	n.a.	3.4	5.9	6.7
+	+	-	4.3	0	0
-	+	+	19.7	17.7	0
-	-	n.a.	29.9	50	80
-	+	-	5.1	2.9	0

Table 3. Functional deficits in antigen presentation in tumor cells of classical Hodgkin lymphoma, diffuse large B-cell lymphoma and primary central nervous system lymphoma patients

Antigen presentation capability	cHL %	NLPHL %	DLBCL %	PCNSL %	Testis %
'Normal'	12.4	90.4	37.6	23.5	13.3
HLA class I dysfunction	17.6	4.8	19.7	17.7	0
HLA class II dysfunction	24.1	0	7.7	5.9	6.7
HLA class I and II dysfunction	45.9	4.8	35.0	52.9	80

Notes: Membraneous expression of HLA class I and class II with cytoplasmic HLA-DM. HLA class I dysfunction indicates loss of membraneous HLA class I staining with preserved membraneous HLA class II and cytoplasmic HLA-DM. HLA class II dysfunction refers to loss of HLA class II or loss of HLA-DM with preserved membraneous HLA class I. HLA class I and II dysfunction indicates loss of membraneous HLA class I combined with either loss of membraneous class II or loss of HLA-DM

Figures

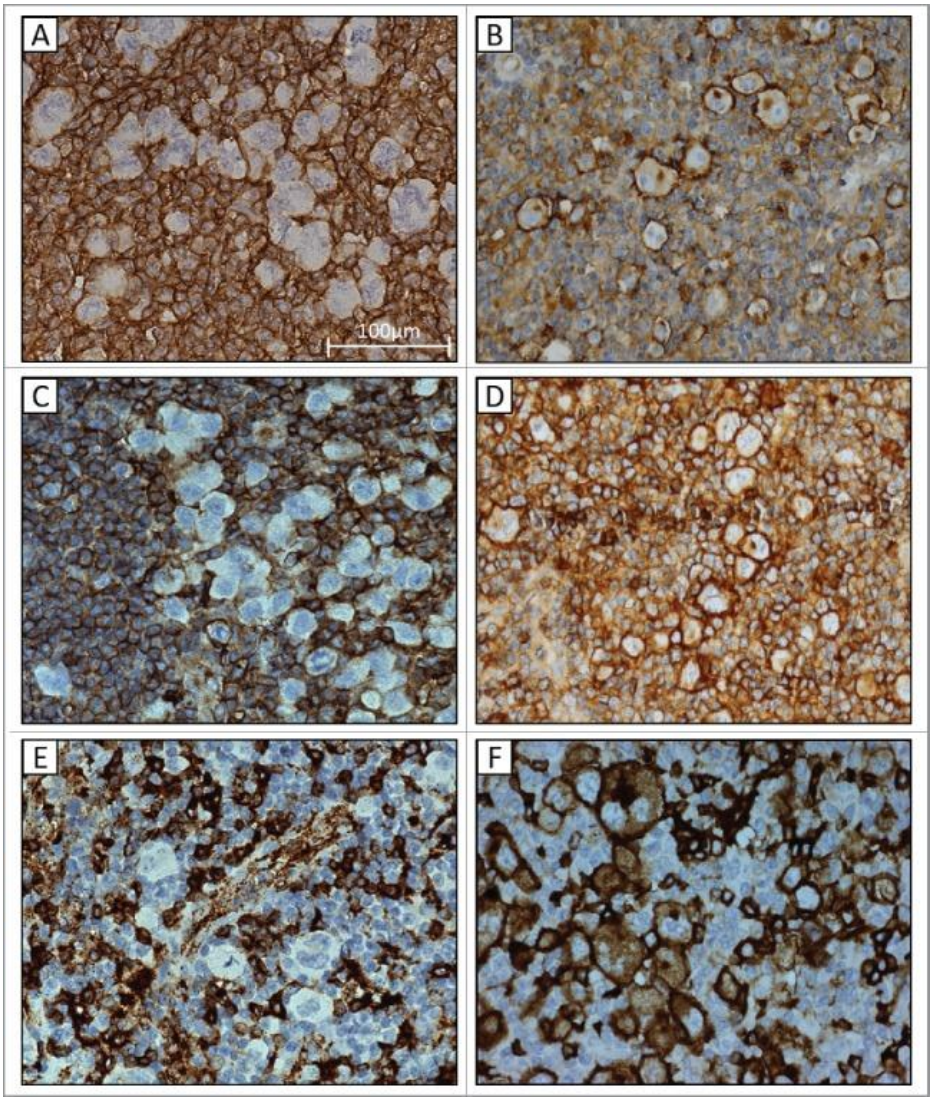


Figure 1. Immunohistochemical staining patterns in formalin fixed embedded classical Hodgkin lymphoma tissue for HLA class I, B2M and HLA class II. (A and B) negative and positive membranous staining for HLA class I heavy chains. (C and D) negative and positive membranous staining for B2M. (E and F) negative and positive membranous staining for HLA class II. 40x

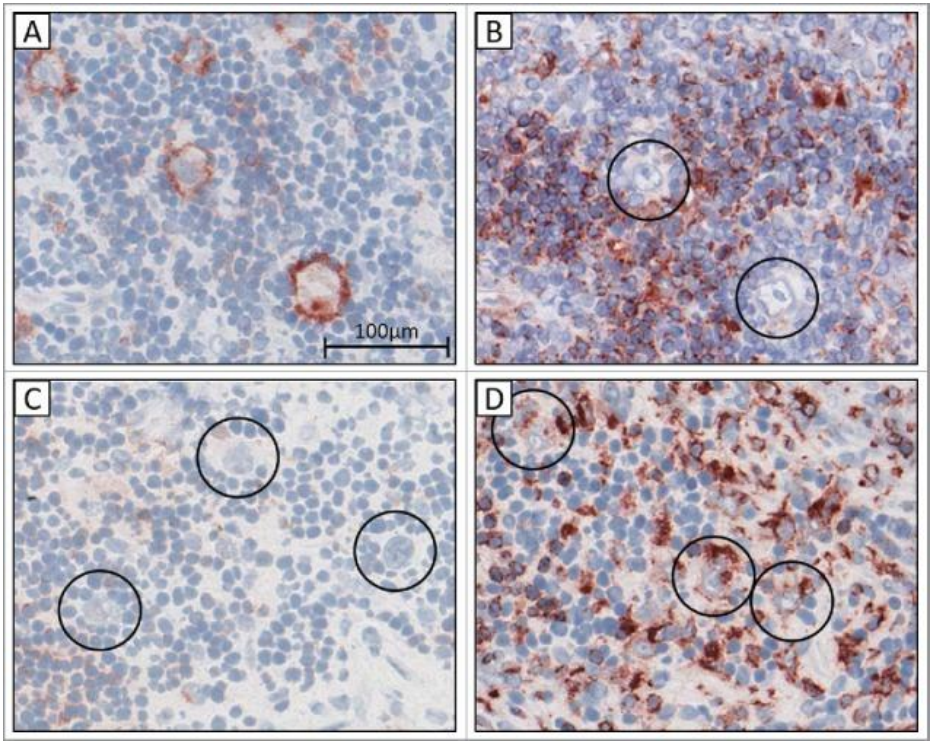


Figure 2. CLIP and HLA-DM immunohistochemistry in frozen classical Hodgkin lymphoma tissue from two representative patients. (A) aberrant membranous CLIP staining in (B) the absence of HLA-DM. (C) normal absence of membranous CLIP staining in (D) presence of cytoplasmic HLA-DM staining. Circles indicate tumor cells. 40x.

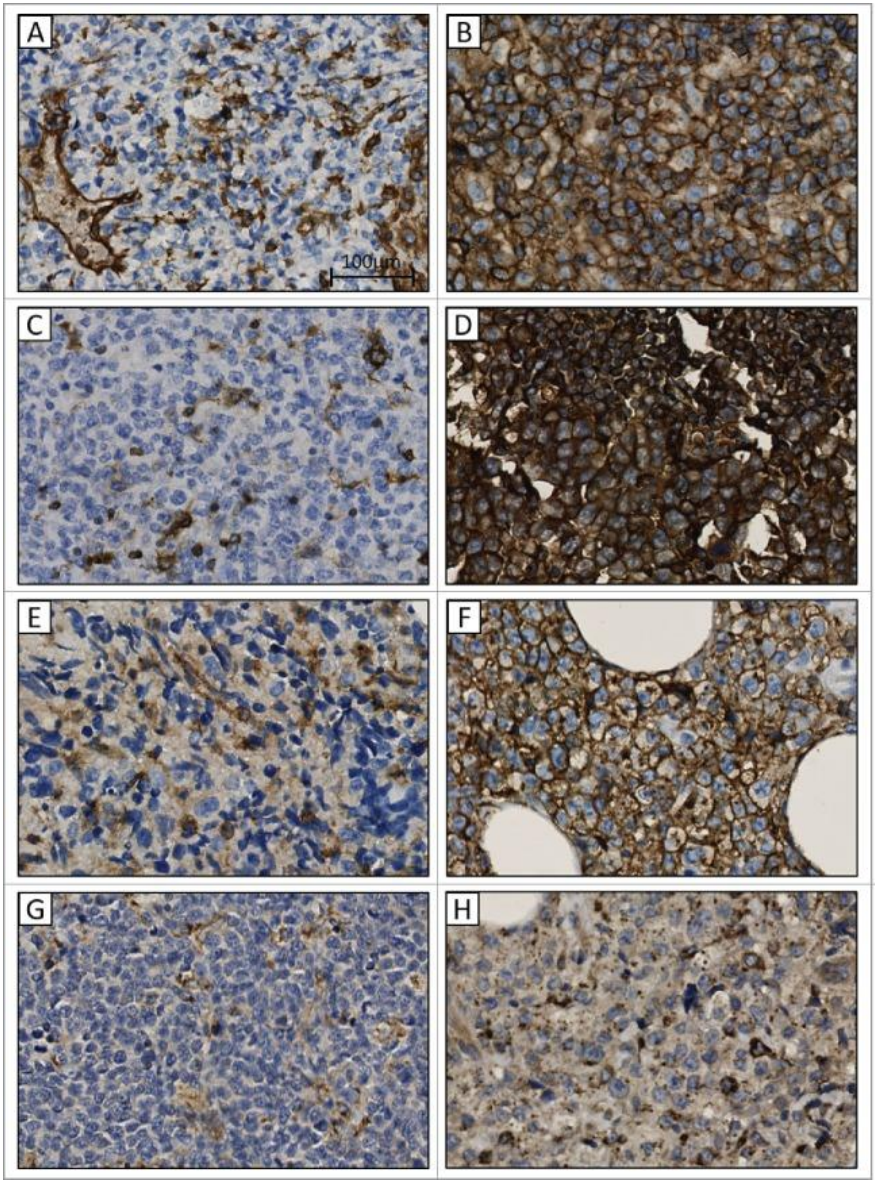
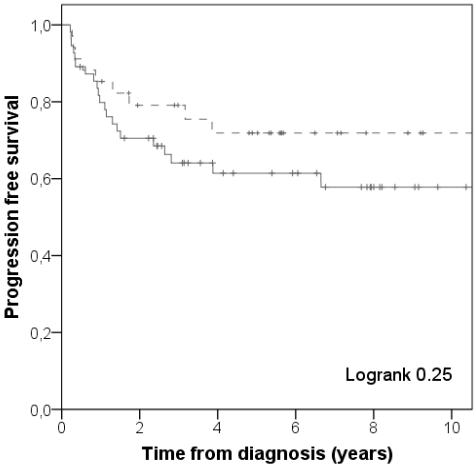


Figure 3: Immunohistochemical staining patterns in formalin fixed paraffin embedded diffuse large B-cell lymphoma tissue for HLA class I, B2M, HLA class II and HLA-DM. (A and B) negative and positive membranous staining for HLA class I heavy chains. (C and D) negative and positive membranous staining for B2M. (E and F) negative and positive membranous staining for HLA class II. (G and H) negative and positive cytoplasmic staining for HLA-DM. 40x.

Supplementary

Supplementary table 1. Clinical characteristics of the 117 patients with diffuse large B-cell lymphoma stained for HLA class I, HLA class II and HLA-DM

	Total (n = 117)	**Normal HLA** (n = 44)	**Aberrant HLA** (n = 73)	**p-value**
Gender				
Male % (n)	60.0 (70)	56.8 (25)	61.6 (45)	0.70
Female % (n)	40.0 (47)	43.2 (19)	36.4 (28)	
Median age (range)	65 (15-92)	63 (15-92)	66 (19-92)	0.90
Ann Arbor #				
Stage I/II % (n)	51.8 (57)	55.8 (24)	49.3 (33)	0.74
Stage III/IV % (n)	48.2 (53)	44.2 (19)	50.7 (34)	
Localisation				
Nodal % (n)	62.4 (73)	66.0 (29)	57.1 (44)	0.61
Extranodal % (n)	37.6 (44)	34.0 (15)	42.9 (33)	
IPI-score *				
Low (0-1) % (n)	46.1 (53)	45.4 (20)	46.5 (33)	
Intermediate (2-3) % (n)	47.8 (55)	52.3 (23)	43.9 (32)	0.41
High (4-5) % (n)	6.1 (7)	2.3 (1)	8.2 (6)	



Supplementary figure 1: Progression free survival Kaplan-Meier plot of diffuse large B-cell lymphoma patients treated with R-CHOP, comparing aberrant to normal tumor cell HLA expression.

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Chapter 5

Combined loss of HLA I and HLA II expression is more common in the non-GCB type of diffuse large B cell lymphoma

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Introduction

As an immune escape mechanism, tumour cells may down-regulate expression of human leucocyte antigens (HLA). Loss of HLA classes I and II has been described in various subtypes of diffuse large B cell lymphoma (DLBCL), including the not otherwise specified (NOS) subgroup.[1,2] We analysed HLA classes I and II expression in DLBCL-NOS to investigate whether there is an association between HLA expression, cell of origin (COO) and the recently reported FoxP1 expression in non-GCB DLBCL.[3]

Material and methods

Case selection

We selected 77 cases of primary DLBCL-NOS diagnosed between 1992 and 2012 in the University Medical Centre Groningen. The cases were reviewed, based on the 2008 WHO Classification. Cases with a presentation at extranodal sites in the skin, bone, CNS, testis and GI tract were excluded. In addition, patients history was checked for an indolent lymphoma.

Immunohistochemistry

Formalin fixed, paraffin embedded tissues were stained for HLA class I (clone HC10, reacting with HLA-B, HLA-C and HLA-A (gift of J. Neefjes, NKI Amsterdam), HLA class II (clone CR3/43, reacting with HLA DP, DQ, DR, Dako (Agilent Technologies, Santa Clara, CA USA)), FOXP1 (Abcam, Cambridge, UK), CD10 (clone SP67 (Ventana, Roche, Basel, Switzerland)), BCL6 (clone CI191E/A8 (Ventana)) and MUM1 (clone MUM-1p (Abcam)). All cases were independently scored by two investigators (LEM and PMK). For evaluation of HLA class I and class II expression, other cells than tumor cells were used as internal negative and positive controls. Tumor cells were scored as homogeneously positive or negative or partially negative when distinct areas showed lack of expression. Hans criteria were used to classify cases as GCB or ABC. Accordingly, for CD10, BCL6 and MUM1 a threshold of >30% positive tumor cells was used. FOXP1 was scored in ten-parts of 0-100% positive tumor cells and considered positive when ≥80% of the tumor cells stained positive (S1 and S3). Sixteen cases could not be scored for FOXP1 due to technical problems.

Statistics

A two-sided Fisher's exact test was performed. P values <0.01 were considered significant.

Results

Seventy-seven cases of DLBCL-NOS with an almost exclusively nodal presentation were classified for COO by immunohistochemistry (IHC) according to the Hans algorithm as germinal centre B (GCB) cell-type (n = 42) or non-GCB-type (n = 35) (Table 1). Clinical data available from 67 cases indicated a histologically confirmed pre-existent or concurrent indolent lymphoma in 16 patients, i.e. 13 follicular lymphoma, two marginal zone lymphoma and one chronic lymphocytic leukaemia. Expression of HLA and FoxP1 was assessed by IHC; methods are described in the Supporting Information.

Significant differences (P < 0.01) in HLA loss were observed between the COO categories in the total group: loss of HLA class I in 51% of non-GCB versus 21% of GCB, of HLA class II in 37% of non-GCB versus 10% of GCB and combined loss in 34% of non-GCB versus 5% of GCB (Figure 1A).

In the total group, 35% of the cases showed loss of HLA class I expression, 22% showed loss of HLA class II and 18% showed loss of both, whereas 25% retained expression of both HLA classes I and II. Two lymphomas with partial loss of HLA class I and two other lymphomas with partial loss of HLA class II were considered HLA-negative.

In 51 *de-novo* cases loss of HLA class I was observed in 21 (41%) and loss of HLA class II in 16 (31%) cases. In 16 transformed cases, expression of HLA class I was lost in 5 (31%; Figure S2), two cases of which showed additional loss of class II (13%). As transformation from follicular lymphoma is associated with the GCB-type, we studied GCB-type DLBCL separately, and found loss of HLA class I in five of 14 transformed cases (36%) versus four of 28 *de-novo* GCB cases (14%) (P = 0.13). In consequence, the differences for HLA class I between the COO subtypes were even more pronounced after exclusion of transformed cases: 55% of non-GCB versus 14% of GCB cases showed loss of expression ($P \leq 0.01$). Thus, these results were not influenced by the inclusion of 16 cases with a history of follicular lymphoma or other types of indolent lymphoma which, in line with the literature, were almost exclusively of GCB-type.

Discussion

Brown *et al*. suggested that FoxP1, a protein associated with non-GCB-type DLBCL, is related inversely with HLA class II expression.[4] A high expression of FoxP1 with a low expression of HLA class II expression was observed in normal pre-plasma cells and non-GCB-type DLBCL maturing to plasmablasts.[5] This is in line with data from the group of Rimsza *et al*., suggesting that loss of HLA class II is seen in DLBCL cases maturing into plasmablastic lymphoma.[2,6] We studied the correlation between FoxP1 expression and HLA class II expression in 61 DLBCL cases (Figures S1 and S3). FoxP1 expression was not associated significantly with non-GCB-type DLBCL (Table 1; $P = 0.08$). We did not find an association between FoxP1 and HLA class II expression: loss of HLA class II was associated very weakly with FoxP1 expression in both non-GCB- and GCB-type DLBCL. However, the majority of cases retained HLA class II expression while expressing high FoxP1 levels in both categories and vice versa; not all cases with low HLA class II showed high FoxP1 expression (Figure 1B,C). Of note, high expression of FoxP1 and HLA class II expression were excluded from analysis in the original study.[4] Furthermore, while most FoxP1-positive cases showed homogeneous staining of the tumour cells and only a few cases showed partial loss of HLA class II expression, a qualitative (intensity of staining) analysis of both proteins is not accomplished easily by immunohistochemistry.

Conclusion

In conclusion, we show that loss of HLA class I and/or HLA class II, in particular the double-negative signature, is much more common in non-GCB-type DLBCL than in GCB-type DLBCL. Our data support previous reports, focusing on class II expression.[6] The preferential loss of HLA class II expression in non-GCB-type DLBCL cannot be explained by a higher expression of FoxP1 alone.

Tables

Table 1. Features of DLBCL-NOS cases

Characteristic	**Non-GCB** (n = 35)	**GCB** (n = 42)	**P-value**
Male (%)	23 (66)	24 (57)	
Median age (years; range)	66 (9–85)	56 (14–77)	
Ann Arbor stage			
I/II (%)	6 (17)	18 (43)	
III/IV (%)	26 (74)	18 (43)	
Unknown (%)	3 (9)	6 (14)	
IPI			
0–1 (%)	5 (14)	13 (31)	
2–3 (%)	19 (55)	18 (43)	
4–5 (%)	7 (20)	1 (2)	
Unknown (%)	4 (11)	10 (24)	
Transformation[a] (%)	2 (6)	14 (33)	<0.01
HLA			
HLA class I^+ (%)	17 (49)	33 (79)	<0.01
HLA class I^- (%)	17 (49)	8 (19)	
Partial loss HLA class I (%)	1 (2)	1 (2)	
HLA class II^+ (%)	22 (63)	38 (91)	<0.01
HLA class II^- (%)	12 (34)	3 (7)	
Partial loss HLA class II (%)	1 (3)	1 (2)	
Double-negative (%)	12 (34)	2 (5)	<0.01
FoxP1 ≥ 80%$^+$/evaluable cases (%)	18/26 (69)	16/35 (46)	0.08

IPI, International Prognostic Index; HLA, Human leucocyte antigen; FoxP1, Forkhead box protein 1; GCB, Germinal centre B cell; DLBCL-NOS, Diffuse large B cell lymphoma, not otherwise specified. [a] *Transformation from a pre-existent or concurrent indolent lymphoma; see tekst.*

Figures

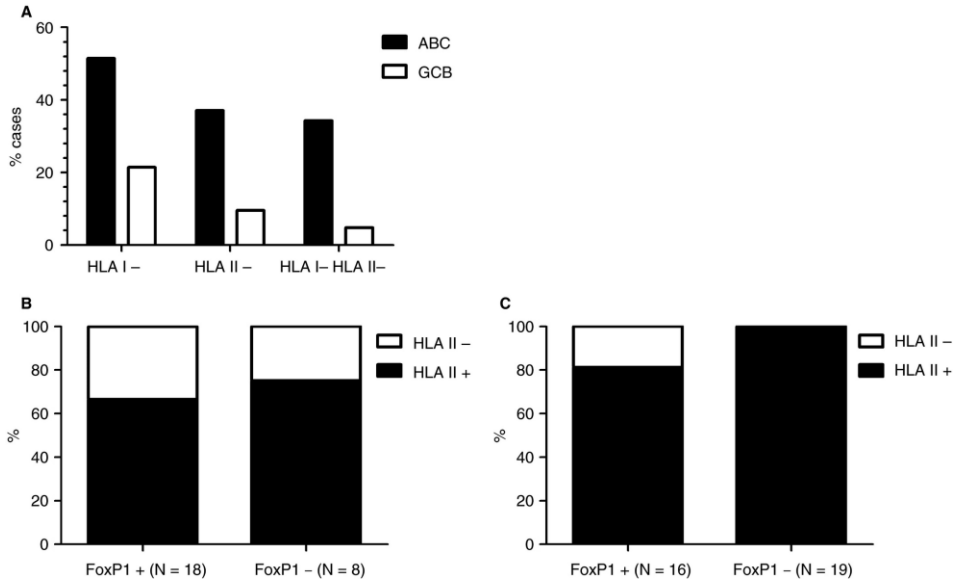
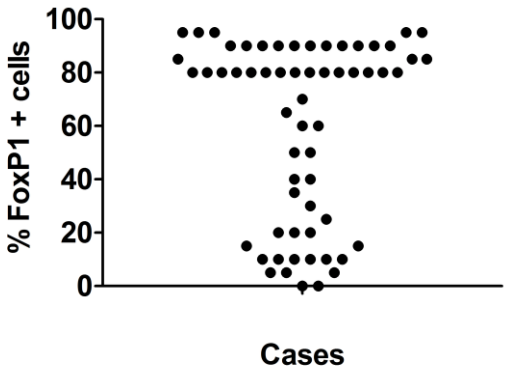
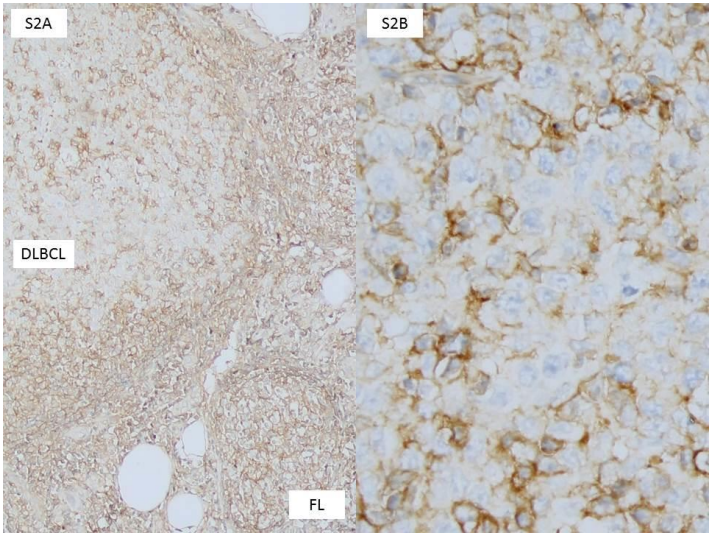


Figure 1: Difference in loss of human leucocyte antigen (HLA) class I and HLA class II between non-germinal centre B cell (GCB) and GCB diffuse large B cell lymphoma (DLBCL). A, Percentage of HLA class I and II negative cases in non-GCB and GCB DLBCL and double-negative cases; B, percentage of FoxP1 positive and negative cases in HLA class II negative and positive non-GCB DLBCL cases; C, percentage of FoxP1 positive and negative cases in HLA class II negative and positive GCB-type DLBCL.

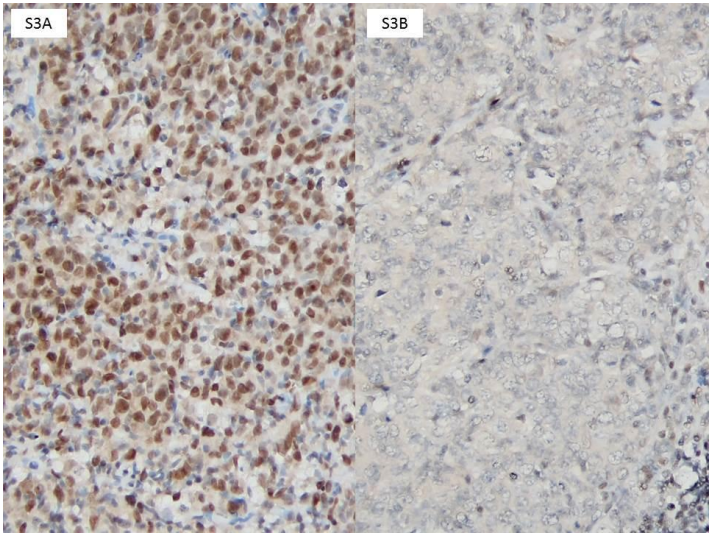
Supplementary



Supplementary figure S1. Percentage of FOXP1 positive cells. A cut-off of ≥80% was used as in the literature,[4] since most cases were below 20% or 80% and higher.



Supplementary figure 2. HLA class I expression in a single case with DLBCL and co-existent follicular lymphoma (FL). The DLBCL shows loss of expression while the FL shows retained expression.



Supplementary figure 3. FoxP1 expression.

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Chapter 6

Relapse in stage I (E) diffuse large B-cell lymphoma

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Abstract

Despite a general favourable outcome in limited stage diffuse large B-cell lymphoma (DLBCL), relapses occur in about 10 to 20% of patients. Prognostic models only partially identify patients at risk for relapse. Moreover, it is not known whether the outcome after such a relapse is similar to the outcome after relapse in advanced stages. From January 2004 through December 2012, all newly diagnosed patients with stage I(E) DLBCL were retrospectively analysed from 2 clinical databases to investigate the relapse pattern and outcome in relation to initial treatment and clinical characteristics. In 126 patients (median age 64 years), histologically confirmed stage I(E) DLBCL was diagnosed. With a median follow-up of 53 months (range 5-132 months), 1 progressive disease and 18 relapses occurred. The 5-year time to tumour progression and disease-specific survival were 85% (95% CI 79-91%) and 92% (95% CI 87%-97%), respectively. We observed no significant difference in relapse localization, time to tumour progression, and disease-specific survival between patients treated with abbreviated R-CHOP plus involved field radiotherapy or with 6 to 8 cycles of R-CHOP. Analysis of relapses showed relapse >5 years after initial treatment (late relapse) in 5 of 19 patients (26%). Six of 19 patients (32%) had central nervous system relapse. Three of 11 relapsed cases available for analysis (28%) showed an MYC translocation, suggesting an overrepresentation in the relapse group. Outcome of patients with a relapse was poor with a median survival after relapse of 8 months. Only 1 patient (5%) underwent successful autologous stem cell transplantation. To improve outcome in these patients, early identification of new biological factors such as a MYC translocation or a high risk for CNS dissemination might be helpful. Moreover, treatment of any relapse after stage I disease should be taken seriously. Salvage treatment should be similar to relapses after advanced DLBCL.

Introduction

Diffuse large B-cell lymphoma (DLBCL) accounts for 25 to 30% of adult non-Hodgkin lymphomas.[1] Twenty-five to 40% of patients present with limited stage disease, defined as stages I and II according to the Ann Arbor classification.

Until the beginning of this century, optimal treatment for limited stage DLBCL used to consist of 3 cycles of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy plus involved field radiotherapy (IFRT). This combined modality approach resulted in a significantly better overall survival (OS) than treatment with 8 cycles of CHOP alone.[2] The addition of rituximab to CHOP has increased OS with 10-15% in both limited stage and advanced stage DLBCL.[3-5] Apparently, this became for many haematologists a reason to refrain from consolidation IFRT for patients with stage I and II.[6] Although randomized controlled trials are lacking, a very large registry study covering >59,000 patients strongly suggested that combined modalitytherapy was associated with better OS, even in the rituximab era.[6] Despite the generally favourable outcome, relapses still occur in 10 to 20% of patients with limited stage DLBCL and 5-year OS ranges between 75% and 94%, which suggests that salvage of relapses is frequently unsuccessful.[6-10] Clinical prognostic models only partially identify patients at risk for relapse.[2,11] Biological tumour characteristics such as cell of origin and especially presence of MYC translocation have prognostic significance in DLBCL.[12,13]

We decided to analyse in an observational cohort study the relapses of patients with stage I(E) DLBCL focusing on (1) initial therapy (only R-CHOP vs. combined modality treatment), (2) clinical characteristics and risk profile of the patient, (3) patterns of relapse, (4) if available the presence of MYC breaks, and (5) the final outcome after treatment. To this end, we used 2 large databases in the northern part of the Netherlands, thereby avoiding trial-based selection and better approaching real life observation.

Material and methods

Study design and patient identification

Clinical data on all consecutive patients with histologically confirmed stage I(E) DLBCL diagnosed during an 8-year period from January 1, 2004 through December 31, 2012 were retrieved from 2 clinical databases from 5 medical centres and 1 academic medical centre. The combined databases are representative of the incidence, characteristics, and treatments of patients in the northern part of the Netherlands. Patients should have received at least 1 cycle of R-CHOP.

Primary coetaneous, central nervous system (CNS) large B-cell lymphoma, primary mediastinal B-cell lymphoma, and immunodeficiency lymphomas were excluded. At diagnosis, patients were staged by fludeoxyglucose positron emission tomography (18FDG PET) and/or computed tomography (CT) scans. The stage-adjusted IPI and CNS IPI were used to stratify patients.[2,14] Pathological review was performed by experienced haematopathologists (RK and AD). Approval for this observational study was obtained from the Medical Ethics Review Committee from Medical Centre Leeuwarden. Informed consent was waived in accordance with Dutch regulations. after 5 years were designated as late relapses.[16,17] Follow-up was completed until December 2015.

MYC fluorescence in situ hybridization analysis

For evaluation of a MYC translocation, formalin-fixed paraffinembedded tissue blocks were collected of the relapsed DLBCL cases. Interphase fluorescence in situ hybridization (FISH) was performed on 3-µm-thick whole tissue sections of the primary tumour as previously described by using Vysis break apart probes (Abbot Technologies).[18]

Statistical analysis

Duration of follow-up was calculated for all patients alive. The primary endpoints were OS, disease-specific survival (DSS), time to tumour progression (TTP), and survival after relapse. Overall survival was defined as time from diagnosis until death (from any cause), DSS as the time from diagnosis until death as a consequence of DLBCL, TTP as the time from diagnosis until relapse or progression, and survival after relapse as the time from relapse until death (from any cause).[15] Survival curves were estimated according to the Kaplan-Meier method. Between-group differences in DSS and TTP were evaluated by using the log-rank test. All categorical variables were expressed as counts and percentages. Where applicable, differences between groups were evaluated by chi-square for binary variables and independent t tests for continuous variables. Cox regression was used for univariate analysis. Given the low incidence of events no multivariate analysis was performed. A 2-tailed P value of less than.05 indicated statistical significance. All analyses were performed by using IBM SPSS Statistics version 22.

Results

Clinical characteristics

A total of 126 patients with a median age of 64 years were eligible for analysis. At presentation, 41% (n = 50) of patients had a nodal localization; extranodal sites consisted of gastrointestinal (20%, n = 25), bone (10%, n = 13), and nasopharyngeal localization (11%, n = 14). Other sites encompassed the remaining 18%, i.e., testis (n = 7), thyroid (n = 5), breast (n = 5), and salivary glands (n = 2) (Figure SS1). High stage-adjusted IPI was observed in 19% (n = 23) of patients. Central nervous system-IPI 0-1 (low risk), 2 to 3 (intermediate risk), and >3 (high risk) were observed in 52% (n = 66), 47% (n = 59), and 1% (n = 1), respectively.

Treatment

Of the 126 patients 97% (n = 122) completed, at least 3 cycles of R-CHOP were evaluable for comparison between treatment arms, e.g., combined modality (68%, n = 83) or R-CHOP alone (32%, n = 39); see Figure 1 and Table 1. In patients receiving R-CHOP, the number of cycles was reduced in 8% of patients (n = 3) because of previous tumour resection and treatment-related toxicity. The cumulative dosage of IFRT was 30 to 40 Gray. All patients with testicular localization received CNS prophylaxis with intrathecal methotrexate.

Clinical and biological characteristics of relapse

One patient had PD during the first 3 cycles of R-CHOP, and 18 patients experienced a relapse. Of these 18 patients, 28% of patients (n = 5) had a relapse more than 5 years after diagnosis: 3 of 14 patients treated with abbreviated R-CHOP plus IFRT and 2 of 4 patients treated with R-CHOP (p 0.52) (Figure 1). In 79% of cases (n = 15), the relapse/progression occurred at a site distant from the initial tumour localization. In 32% of cases (n = 6), this involved the CNS, with either meningeal and/or parenchymal localization. Two of the CNS relapses occurred in patients with a testicular lymphoma, despite CNS prophylaxis. In the remaining cases, the primary tumour had nodal (n = 2) and nasopharyngeal localizations (n = 2) (Figure 2). The initial calculatedCNS-IPIwas low (n = 3) and intermediate (n = 3). In addition, 1 patient with initial nodal localization had a testicular relapse, another sanctuary site. Of the 19 relapsed/progressive DLBCL, 58% of cases (n = 11) had tissue blocks with sufficient tumour material for MYC-FISH analysis. In 28% of cases (n = 3), an MYC translocation could be demonstrated. All these MYC positive DLBCL were observed in patients with an early relapse. None of the evaluable CNS relapses had a MYC translocation (Figure 2).

Patient outcome

The median duration of follow-up of the 126 patients was 53 months (range 5-132). Twenty-seven patients died (11 of relapse; 16 unrelated). The 5-year TTP, DSS, and OS for the entire cohort were 85% (95% CI 79-91%), 92% (95% CI 87%-97%), and 80% (95% CI 73-86%),

respectively (Figure 3A and B). In univariate analysis of 122 patients completing therapy, age had the strongest association with shorter TTP (Table 2). Age > 60 years and elevated LDH, both composites of stage adjusted IPI, were associated with a shorter DSS (Table 2). Localization and treatment regimen were not found to be prognostic factors (Figure S2A). Univariate analysis showed that age > 60 years (HR 9.1,95% CI 2.1-38, P < .01), but not localization, LDH, or treatment regimen was associated with a shorter OS (Figure S2B).

Treatment after relapse

The median age of relapsing patients was 77 years (range 41-83). The median survival after relapse was 8 months (Figure 3C). Treatment of patients who relapsed consisted of rituximab, dexamethasone, cytarabine, and cisplatin salvage chemotherapy (16%, n = 3), retreatment with R-CHOP (10%, n = 2), palliative chemotherapy (16%, n = 3), radiotherapy (32%, n = 6), and palliative care (26%, n = 5). Only 1 patient (5%) underwent high dose chemotherapy followed by autologous stem cell transplantation as part of the salvage regimen. A second remission was achieved in 3 of 5 (60%) patients receiving curative chemotherapy, in 1 patient (33%) treated with "intended" palliative chemotherapy, and in 2 patients (33%) treated with radiotherapy.
One-year survival after relapse for patients receiving salvage radiotherapy or chemotherapy was 53% (95% CI 27-79%).

Discussion

In this population-based cohort study with a long median follow-up, we followed all consecutive patients with newly diagnosed DLBCL stage I(E) during an 8-year period. We observed no differences in relapse localization, TTP, and DSS between patients treated with abbreviated R-CHOP plus IFRT and R-CHOP only. Obviously, this was not a randomized comparison but a reflection of real-life approaches. Patients with extranodal disease more frequently received R-CHOP, reflecting physicians' choice to avoid radiotherapy-induced toxicity. By looking into the characteristics of relapsed patients, we made several observations that offer a reason for the occurrence of relapses in these good risk patients.

Regardless of initial therapy, one-third of relapses (26%) occurred more than 5 years after therapy. Although late relapses have been observed in the pre-rituximab era,[17] we and others observed these late relapses in patients with limited stage DLBCL treated with rituximab as well.[16] Although clonal relationship in late relapses is established, the biology underlying the long interval remains unclear.[19] Most relapses (73%, n = 15) arose at distant sites, indicative of

good local tumour control with either abbreviated R-CHOP plus radiotherapy or R-CHOP. In nearly one-third of relapses, there was CNS involvement. Because rituximab and CHOP have only limited activity in the CNS, it is unlikely that either of the treatment regimens will prevent the CNS relapses.[20] Even when initial CNS prophylaxis with intrathecal MTX is provided, CNS relapses can occur as illustrated by 2 patients with lymphoma of the testes in our study.[21] Recently, the CNS-IPI as risk model for CNS relapse in patients with DLBCL was established to identify patients at highest risk for CNS relapse.[14] In patients with a low-risk CNS-IPI, less than 1% showed a CNS relapse.

However, as shown in our study, with stage I(E) DLBCL, 5% of 126 patients with initial low-risk CNS-IPI had a CNS relapse. It was recently reported that in contrast to advanced stage DLBCL, the cell-of-origin is not prognostic in limited stage disease.[9,13] To impact prognosis of these good risk patients, a biomarker, such as MYC breaks, might be helpful.[12] We found an MYC translocation in 3 of 11 evaluable relapsed cases. It is plausible that in the relapsed setting, 15 to 20% of DLBCL harbour an MYC translocation.[22] Although the number of analysed patients is low, we found no MYC positive DLBCL in late relapses. Despite an increased risk of CNS dissemination in MYC positive DLBCL, no translocation was detected in the 2 evaluable CNS relapses.[23] Combined analysis showed that two-third of relapses could be assigned to either a late relapse, CNS relapse, or MYC positive DLBCL.

In general, outcome of patients with relapsed or refractory DLBCL is very poor, with the exception of more favourable outcome in relapses more than 1 year after treatment.[24] We observed a similar poor outcome in patients with relapsed stage I(E) DLBCL. This can partially be explained by the old age of the relapsed patients, limiting therapeutic options. Because the median age of patients with a DLBCL in the general population is 68 years, this is an observation in line with general practice. Furthermore, treatment options for relapsed DLBCL in the CNS are limited and patients with an MYC translocation tend to have a poor response to salvage chemotherapy.]25,26] Salvage therapy for a relapse after stage I(E) disease is not trivial and emphasizes the necessity to improve first line treatment in these patients as well.

Conclusion

Despite a general favourable outcome in stage I(E) DLBCL, 15% of patients relapsed despite previous R-CHOP therapy and survival after relapse was short. Analysis of relapses showed that more than half of cases could be assigned to either a late relapse or CNS relapse. Interestingly, there is a suggestion that MYC positive DLBCL was overrepresented in the relapse group. Although numbers are small, our results emphasize the necessity to improve first line treatment in these patients as well. Any relapse after stage I disease should be taken seriously, and patients need similar intensive salvage therapy as after advance stage disease relapses.

Tables

Table 1. Clinical characteristics of 122 patients with stage I (E) diffuse large B-cell lymphoma who completed therapy according to treatment regimen.

	Total (n = 122)	**R-CHOP** (n = 39)	**Abb. R-CHOP +** IFRT (n = 83)	**p-value**
Gender				
Male (%)	68 (56)	27 (69)	41 (49)	0.04
Female (%)	54 (44)	12 (31)	42 (51)	
Median age (range)	64 (15-87)	63 (15-83)	66 (28-87)	0.43
Age < 60y (%)	48 (39)	16 (41)	32 (39)	0.79
Age > 60 (%)	74 (61)	23 (59)	51 (61)	
Localization				
Nodal (%)	50 (41)	8 (21)	42 (51)	< 0.01
Extranodal (%)	72 (59)	31 (79)	41 (49)	
Performance				
WHO < 2 (%)	118 (97)	39 (100)	79 (95)	0.37
WHO ≥ 2 (%)	4 (3)	0 (0)	4 (5)	
LDH				
Normal (%)	99 (81)	26 (67)	73 (88)	0.01
Elevated (%)	23 (19)	13 (33)	10 (12)	
IPI				
0-1 (%)	64 (52)	15 (39)	49 (59)	0.03
2-3 (%)	58 (48)	24 (62)	34 (41)	
Stage-adjusted IPI				
0-1 (%)	99 (81)	29 (74)	70 (84)	0.19
2-3 (%)	23 (19)	10 (26)	13 (16)	

Table 2. Univariate analysis of factors in time to tumor progression (TTP) and disease specific survival (DSS) of 122 stage I (E) diffuse large B-cell lymphoma patients who completed therapy.

	%	Hazard ratio TTP	95% CI	p-value	Hazard ratio DSS	95% CI	p-value
Gender							
Male	56		Reference			Reference	
Female	44	0.97	0.4 - 2.5	0.94	0.94	0.3 - 3.1	0.92
Age							
< 60 years	39		Reference			Reference	
> 60 years	61	4.1	1.2 - 14	0.03	3.6	0.8 - 17	0.06
Localization							
Nodal	41		Reference			Reference	
Extranodal	59	1.2	0.5 - 3.0	0.72	1.2	0.3 – 4.4	0.80
LDH							
Normal	81		Reference			Reference	
Elevated	19	2.0	0.7 – 5.8	0.19	4.4	1.3 - 15	0.02
IPI							
0-1	52		Reference			Reference	
2-3	48	2.7	1.0 – 7.2	0.05	3.8	1.0 - 15	0.05
Stage adjusted IPI							
0-1	81		Reference			Reference	
2-3	19	2.7	1.0 – 7.5	0.05	4.5	1.3 - 15	0.02
Treatment regimen							
R-CHOP + IFRT	68		Reference			Reference	
R-CHOP	32	0.66	0.2 – 2.0	0.47	2.0	0.6 - 6.9	0.28

Figures

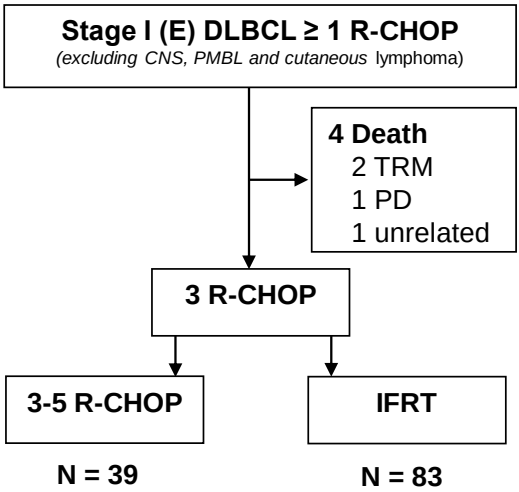


Figure 1. Schematic representation of the 126 patients with a stage I(E) diffuse large B-cell lymphoma according to treatment regimen. One hundred twenty-two patients completed therapy. Four patients died before completing 3 cycles of (R)CHOP. Abbreviations: TRM, treatment-related mortality; PD, progressive disease

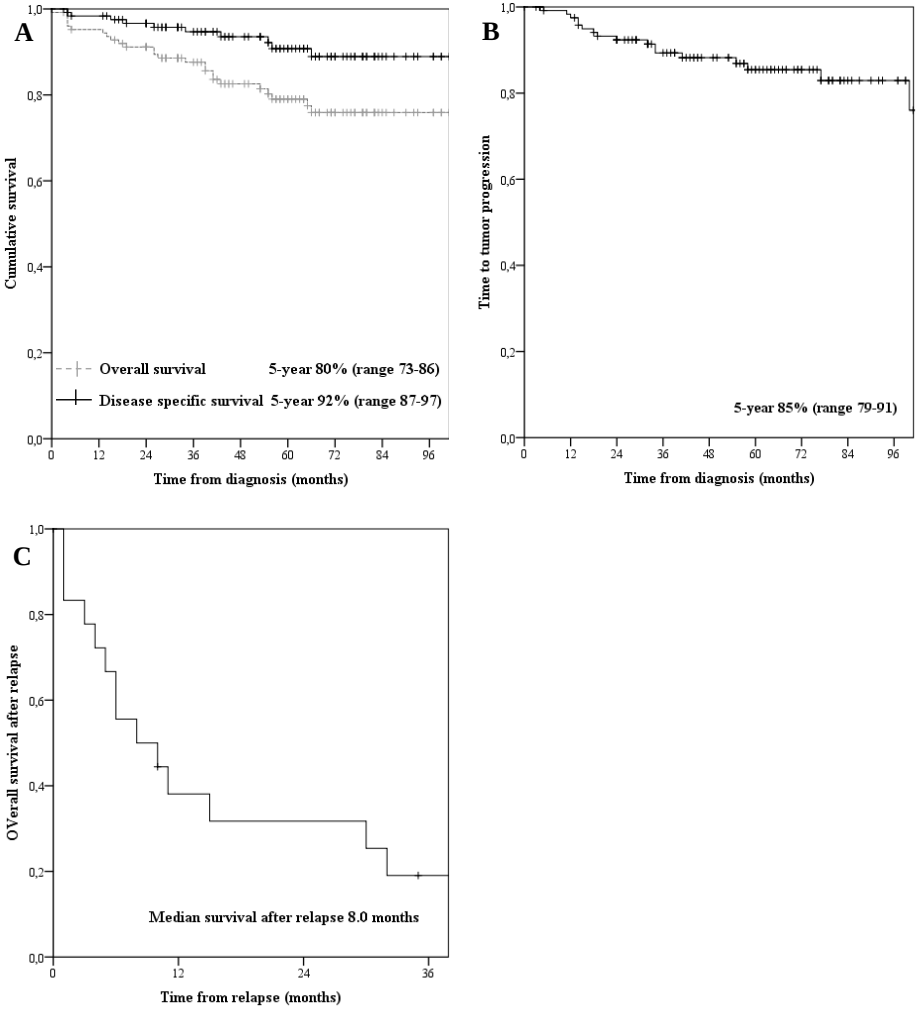


Figure 2. (A) Kaplan Meier curves for overall survival (OS) and disease-specific survival (DSS) of the 126 patients with a stage I(E) diffuse large B cell lymphoma (DLBCL). The 5-year OS and DSS are 80 ± 6 and 92 ± 5%, respectively. (B) Kaplan Meier curve for time to treatment failure (TTF) of the 126 patients with a stage I (E) DLBCL. At 5 years, the TTF was 85 ± 6%. (C) Kaplan Meier curve for survival after relapse of 19 patients with a relapsed stage I(E) DLBCL. Median OS is 8.0 months

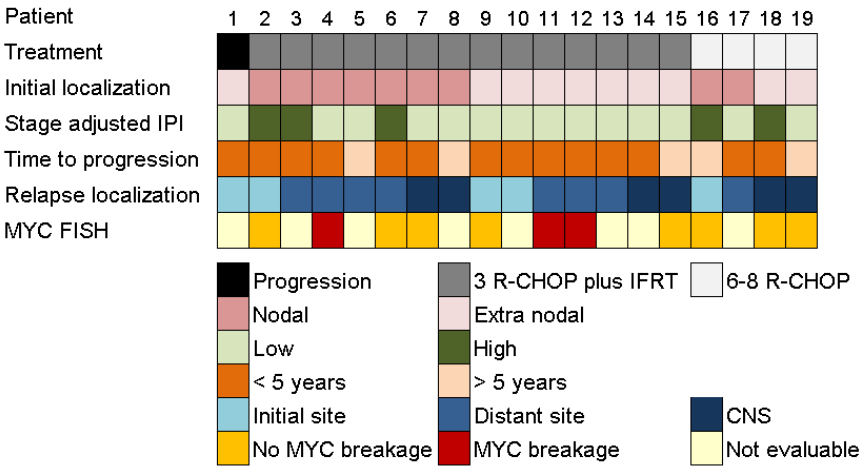
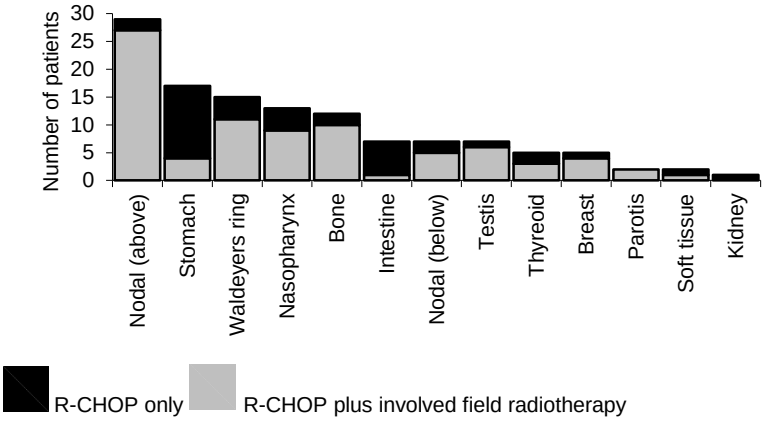
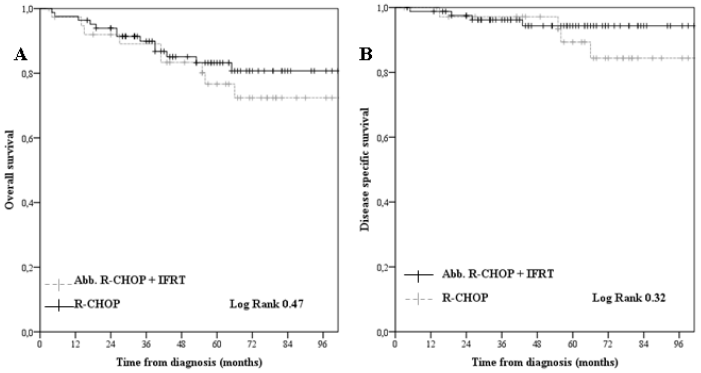


Figure 3. Schematic overview of clinical and biological characteristics of the 19 patients with a stage I(E) diffuse large B-cell lymphoma who progressed during or relapsed after treatment. Patients are categorized according to the type of treatment received.

Supplementary



Supplementary figure 1. Type of treatment of the 122 patients with a stage I(E) diffuse large B-cell lymphoma (DLBCL) who completed therapy according to tumour localization. In nodal DLBCL, abbreviated R-CHOP plus involved field radiotherapy is favoured over R-CHOP. In extranodal DLBCL, nearly half of patients received R-CHOP.



Supplementary figure 2. (A) Overall survival (OS) of the 122 patients with a stage I(E) diffuse large B-cell lymphoma (DLBCL) who completed therapy according to treatment regimen. The 5-year OS of patients treated with abbreviated R-CHOP plus involved field radiotherapy and R-CHOP was 85% and 83%, respectively (p 0.47). (B) Disease-specific survival (DSS) for the 122 patients with a stage I(E) DLBCL who completed therapy according to treatment regimen. The 5-year DSS of patients treated with abbreviated R-CHOP plus involved field radiotherapy and R-CHOP was 93% and 93%, respectively (p 0.32).

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Chapter 7

Mutational evolution in relapsed diffuse large B-cell lymphoma

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Abstract

Current genomic models in diffuse large B-cell lymphoma (DLBCL) are based on single tumor biopsies, which might underestimate heterogeneity. Data on mutational evolution largely remains unknown. An exploratory study using whole exome sequencing on paired (primary and relapse) formalin fixed paraffin embedded DLBCL biopsies (*n* = 14) of 6 patients was performed to globally assess the mutational evolution and to identify gene mutations specific for relapse samples from patients treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone. A minority of the mutations detected in the primary sample (median 7.6%, range 4.8–66.2%) could not be detected in the matching relapse sample. Relapsed DLBCL samples showed a mild increase of mutations (median 12.5%, range 9.4–87.6%) as compared to primary tumor biopsies. We identified 264 genes possibly related to therapy resistance, including tyrosine kinases (*n* = 18), (transmembrane) glycoproteins (*n* = 73), and genes involved in the JAK-STAT pathway (*n* = 7). Among the potentially resistance related genes were *PIM1*, *SOCS1*, and *MYC*, which have been reported to convey a risk for treatment failure. In conclusion, we show modest temporal heterogeneity between paired tumor samples with the acquisition of new mutations and identification of genes possibly related to therapy resistance. The mutational evolution could have implications for treatment decisions and development of novel targeted drugs.

Introduction

Diffuse large B-cell lymphoma (DLBCL) accounts for 25–35% of all non-Hodgkin lymphomas (NHL) [1]. For more than 15 years, immune-chemotherapy with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) has been the standard of care [2]. Although the prognosis for patients with low-risk disease is excellent, the 3-years overall survival (OS) for high-risk patients is less than 65% [3]. Patients with primary refractory disease or those who relapse within a year after treatment have an especially poor response to salvage chemotherapy [4,5].

DLBCL is a genetically heterogeneous disease. Based on gene expression profiling (GEP), DLBCL can be subdivided into activated B-cell (ABC-type), germinal center B-cell (GCB-type), and unclassified-type. The first two subtypes reflect the B-cell developmental stages from which DLBCL arises [6]. The prognostic impact of this so-called cell-of-origin (COO) classification based on gene expression profiles (GEP) has been established in multiple studies [6,7]. Several trials are currently investigating the efficacy of adding compounds targeting the putative mutations or deregulated molecular pathways associated with a specific COO class; for example, adding ibrutinib or lenalidomide to R-CHOP therapy in patients with ABC subtype DLBCL [6–8].
Over the last decade, a large number of studies have been published on the mutational landscape of DLBCL, including over 2000 DLBCL cases [9–21]. These studies identified non-synonymous mutations in 30 to 100 genes per case (median 3.3 to 6.6 mutations per megabase) [21]. In total, over 1000 individual mutated genes have been described. The mutational landscape differs between ABC-type and GCB-type, with mutations in *MYD88* and *CD79B* being more common in the ABC-type, and mutations in *EZH2* and *GNA13* being more common in GCB-type [9–21]. Based on the mutational landscapes, DLBCL can be divided into subgroups characterized by genetic alterations in the proximal B-cell receptor, NF-kB signaling, PI3-kinase signaling, anti-apoptotic proteins, DNA damage repair, and immune evasion [20,21].

One of the main challenges for most genomic profiles is to implement them into clinical practice. Due to the high inter-patient heterogeneity of mutations, it has been estimated that for the development of a prognostic model, the mutational landscape of 900 patients would have to be correlated with clinical outcomes [22]. Earlier trials had been underpowered to properly address this question [16,18]. More recently, larger studies have reported on the prognostic impact of genomic risk models [19–21]. Apart from being prognostic, these genomic models can form the basis for biomarker driven treatment strategies [23].

Most of the currently published papers do not take into account genomic evolution of tumors with presence of subclones at different anatomical sites (spatial), evolution over time (temporal), and

dynamics caused by treatment [24]. At this point, data on the clonal evolution of DLBCL is largely absent. Data on sequential biopsies in DLBCL is available for less than 20 patients [15,18]. Thus, despite the large number of DLBCL samples analyzed, the impact of clonal and mutational evolution remains largely unknown. To broaden this knowledge, we performed an exploratory study on paired biopsies (primary versus relapse) to globally assess the mutational evolution, and to identify gene mutations enriched or exclusively present in relapsing patients.

Materials and Methods

Patient Selection

All patients diagnosed with a relapsed or refractory (R/R) DLBCL between 2004 and 2014 at the University Medical Center Groningen (UMCG) or affiliated hospitals were retrieved from the electronic database of the UMCG. Only patients that received 6 to 8 cycles of R-CHOP as first line therapy were included. Patients with post-transplant lymphoproliferative disease, human immunodeficiency virus (HIV) related lymphoma, primary central nervous system lymphoma (CNS), primary testicular lymphoma, primary mediastinal large B-cell lymphoma, or transformed indolent lymphoma were excluded. Of the 61 patients with R/R DLBCL, a histological confirmation of relapse was available for 31 patients. CNS relapse was confirmed by flow cytometry on spinal fluid samples in 7 patients. In the remaining 23 patients, progression was established through imaging modalities. Of the 31 patients with a histological proven relapse, 16 had an excision biopsy sufficient for further analyses (Figure S1). The remainder of the patients had core or bone marrow biopsies insufficient for analysis. Where applicable, archival samples of non-tumor tissue were retrieved for isolation of germ line DNA. Approval for this non interventional study was obtained from the Medical Ethics Review Committee from the University Medical Center Groningen (October 2014). Informed consent was waived in accordance with Dutch regulations The study utilized rest material from patients, the use of which is regulated under the code for good clinical practice in the Netherlands and does not require informed consent in accordance with Dutch regulations.

Pathology Review

Pathology review was performed according to the 2017 “WHO classification of tumors of haematopoietic and lymphoid tissues” on formalin fixed paraffin embedded (FFPE) biopsies by an experienced hemato-pathologist (AD) [1]. For COO classification, raw counts obtained by nanostring gene expression analysis were uploaded at the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) website for COO categorization (https://llmpp.nih.gov/LSO/LYMPHCX/lymphcx_predict.cgi) [7].

Fluorescence in Situ Hybridization

MYC rearrangements were assessed on interphase nuclei on 3 µm thick whole tissue sections of the primary tumor as previously described, with Vysis break apart probes (Abbot technologies, Santa Clara, CA, USA) using fluorescence in situ hybridization (FISH) [25]. All cases with a *MYC* break were also analyzed for BCL2 and BCL6 breaks using Vysis break apart probe assays (Abbot technologies, Santa Clara, CA, USA).

DNA Isolation

In total, 67 FFPE tissue blocks were obtained from 16 DLBCL patients. For whole exome sequencing (WES) we selected tumor samples with at least 50% tumor cells. To yield at least 500 µg DNA, a minimum area of 0.5 cm^2 of tumor cells was obtained from 10 µm thick slides. DNA from FFPE tumor and non-tumor biopsies was isolated using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany), following the protocol of the manufacturer. A standard salt-chloroform protocol was used to isolate DNA from stem cells collected for hematopoietic stem cell transplantation (CD34+ purified cells) from one patient. DNA concentrations were measured by NanoDrop (Thermo Fisher Scientific Inc., Waltham, MA, USA), and DNA quality was evaluated on a 1% agarose gel. After quality control, 59 samples from 15 patients were sent for WES (Figure S1).

Whole Exome Sequencing

Library preparation and whole exome sequencing was carried out by Novogene (Novogene Bioinformatics Technology Co., Ltd, Beijing, China). Library preparation was done using the Agilent SureSelect All Exon V6 kit (Agilent technologies, Santa Clara, CA, USA), starting from 0.5–1.5 µg genomic DNA of tumor and non-tumor samples. Paired-end sequencing with a read length of 2 × 100 nucleotide was performed on Illumina HiSeq2000 (Illumina, Inc., San Diego, CA, USA).

Bioinformatics Approach

The bioinformatics pipeline of the UMCG genome facility was used for data analysis, as described previously [26,27]. Briefly, reads were aligned to the human 1000 genomes reference based on the GRCh37 build using BWA 5.9rc. [28]. Picard tools were used for format conversion and marking duplicate reads. The Genome Analysis Toolkit (GATK1) was used for realignment of insertions and deletions (Indels), and Molgenis Compute 4 for base score quality recalibration (BSQR) [29,30]. Custom scripts in the VCF tools library were used to generate VCF files, variant calling was performed using the GATK unified genotype, and variant annotation using snpeff/snpsift 3.5 with the ensemble release 74 gene annotations (http://www.ensembl.org/index.html), dbNSFP2.3, and GATK with annotations from the Database of Single Nucleotide Polymorphisms (dbSNP) Bethesda (MD), National Center for Biotechnology Information, National Library of Medicine (dbSNP Build ID: 137), and CosmicCodingMuts_v62.

[31–34]. To identify reliable somatic mutations, variants with total reads <20× in either the normal, primary, or resistant samples were excluded. In addition, we excluded all variants with ≥2 mutant reads in the normal sample, as these might represent personal variants. The remaining variants were aligned against the Exome Aggregation Consortium (ExAC) database (Broad Institute, Cambridge, MA; URL: http://exac.broadinstitute.org) to screen for any remaining known single nucleotide polymorphisms. In addition, we removed variants that (1) were present in the Caucasian based 1000-Genome with an allele frequency larger than 0.2%, (2) map in noncoding regions, (3) were synonymous, (4) have a quality score <20, or (5) have a mapping quality <20. Only variants with ≥2 mutant reads were taken into account.

Genes Possibly Related to Therapy Resistance

Variants specific for the R/R, and variants with mutant read frequencies (MAFs) in the resistant samples ≥20% and with a MAF at least two times higher compared to the MAF in the paired primary sample, were indicated as "*possibly related to therapy resistance*", provided the tumor cell percentage in primary and relapse samples was similar.

Results

Patient Characteristics

Of the 59 samples originating from 15 patients sent for WES, library preparation failed in all 6 samples of a single patient. The average sequencing coverage of the remaining patients was insufficient (<20×) in either the primary sample (6 patients), the relapse sample (1 patient), or both samples (1 patient) (Figure S1). In total, WES data of sufficient quality from sequential biopsies was obtained for six patients. For two of the six patients with relapse biopsies from different anatomical sites, both biopsies were taken at the same time for one patient and at different time points for the other patient. Patient and clinicopathological characteristics are summarized in Table 1. A *MYC* and a *BCL6* rearrangement were observed in the primary tumor sample of patient 5. In 5 patients, relapses occurred within 24 months from diagnosis, and one patient had a relapse at 55 months. The five patients that died were all due to lymphoma progression

Quality Control

The median read depth of the WES data of the 14 tumor samples was 85× (range 29–203×) (Figure S2). The median number of non-synonymous coding single nucleotide variants (SNVs) and Indels per genome was 568 (range 77–949), affecting 1896 genes in total. An estimation of the admixture of normal cells based on the mean mutant allele frequency (MAF) of all somatic mutations in the 25–75% interquartile range, as previously described [26], revealed tumor cell percentages ranging from 78 to 92% (median 90%), and is in concordance with the pathologist's (AD) estimation (data not shown). No significant differences were observed between tumor cell

percentages in the primary and relapse samples (Figure S3). The mean read depth of genes frequently mutated in DLBCL was 285× (range 59–1010×) (Table S1). For a few exons, the depth was insufficient to reliably assess the presence of mutations. In particular, this was the case for exon 3 of FOXO1, with no reads in any of the samples.

Commonly Mutated Genes

Fourteen of the 20 genes most frequently mutated in DLBCL according to the Cosmic database (version 86) were mutated in one or more of the cases in our study (Figure 1). We identified 28 genes with mutations in at least 3 patients (Table S2). Functional annotation of these genes showed enrichment for genes involved in antigen presentation, including the human leukocyte antigen (HLA) molecules and immunoglobulin light chains. Mutations in *Suppressor of Cytokine Signaling 1* (*SOCS1*) and Pim-1 Proto-Oncogene (*PIM1)* were observed in 5 out of 6 patients (Figure S4). The patient with a *MYC* rearrangement had 3 missense mutations in exon 2 of the *MYC* gene. Furthermore, four additional *MYC* mutations were found in two patients without a *MYC* rearrangement (Figure S4).

Mutational Evolution

With the exception of one patient, the vast majority of the mutations were shared between the primary and R/R samples. The median percentage of mutations detected in the primary and not in the matching relapse sample was 7.6% (range 4.8–66.2%) (Figure 2, Table S3). The loss of mutations was particularly high in the patient with a late relapse, where 66% of mutations detected in the primary sample could not be detected in the relapse sample. The mean MAF of the mutations only detected in primary samples was 0.15, which is in the lower quartile of the distribution, indicating genomic heterogeneity in the tumor cells.

Relapsed DLBCL samples showed a median increase of non-synonymous mutations of 12.5% (range 9.4–87.6%) as compared to primary tumor biopsies (Figure 2, Table S3). In the two patients with multiple biopsies at relapse, there was a 45.3% and 89.2% concordance for mutations detected only in the relapse samples (Figure 2). There was no significant correlation between the loss or increase in mutations and time until relapse (ρ 0.32; p, 0.71) (Figure S5). Mutations were randomly distributed across the genome (Figure S6).

Of the 354 relapse specific mutations (in 303 genes), 195 (55%) had a MAF <0.2 and were probably subclonal. The remaining 159 (45%) mutations had MAF ≥0.2 and are probably major clone mutations, and thus possibly related to therapy resistance (Supplementary Table 3). In addition, we identified 215 mutations that showed at least a two-fold increase in MAF in the relapse biopsy compared to the primary sample, indicating a possible relation with therapy. The combined set of 374 mutations possibly related to therapy resistance encompassed 264 genes (Figure S7). Functional annotation of these genes revealed 18 tyrosine kinases, 73 (transmembrane) glycoproteins, and 7 genes that are related to the JAK-STAT pathway (Table

S4). Several of the genes with relapse specific mutations are known to be targets for somatic hypermutation, including *BCL2*, *BIRC3*, *BTG2*, *IRF4*, *MYC*, *PIM1*, *SGK1*, and *SOCS1* [21,35]. The most frequently observed base substitution among the relapse specific mutations (C:G > T:A) is a known cyclophosphamide-induced base substitution, and to a lesser extent a canonical Activation-Induced Deaminase (AID) activity dependent substitution (Figure S8) [35,36]. The evolution of the resistance-associated and other mutations in *SOCS1, PIM1*, and *MYC* showed different patterns (Figure 3). The MAF of mutations in *SOCS1* showed moderate increases in relapse samples. Mutations in *PIM1* showed at least two-fold increased MAF in two out of five patients. In the patients with two relapse biopsies, the MAF of *SOCS1* and *PIM1* were similar across the relapse samples (Figure 3A, B). Even in this small cohort, the dynamics of *MYC* mutations showed clear heterogeneity with loss of mutations (n = 3), increase of MAF (n = 1), and gain of mutations (n = 3) in relapse samples (Figure 3C). The MAF of *SOCS1* mutations remains relatively stable. The MAF of mutations in *PIM1* and *MYC* had at least a two-fold increase in 2 of 5 and 2 of 3 patients, respectively.

Discussion

Mutational analysis has expanded the knowledge on the pathogenesis of DLBCL with genomic risk models that can aid future biomarker driven treatment strategies [19–21]. However, mutational analysis from single tumor biopsies will underestimate the true genomic landscape of tumors due to the presence of inter- and intra-tumor heterogeneity, natural clonal evolution, and therapy related changes [24,37]. Through targeted sequencing of the variable, diversity and joining (VDJ)-segments of the immunoglobulin heavy chain, two DLBCL relapse models have been observed: A late (linear) model and early (divergent) model [15]. Data on mutational evolution in DLBCL remains largely unknown due to the lack of tumor biopsies [18]. In the current study, mutation profiles in relapsed DLBCL were analyzed by pair-wise comparison of primary tumor and relapse samples. Despite the limited number of patients with informative WES data, several compelling observations were made.

First, a median of 12.5% of mutations detected in relapse samples was not detected in the primary samples. Our findings are in line with a previous study showing >80% concordance of mutations in 6 out 7 paired R/R samples [38]. However, in 2 of 6 cases we observed a gain >20% in mutations in the relapse sample. These might be truly relapse specific, or failed detections in the primary sample due to heterogeneity. Based on phylogenetic trees by somatic hypermutation and mutational analysis, an increased number of mutations was proposed to be the result of an early divergent relapse clone [15]. The change in mutational load of relapses might be bigger in the presence of spatial heterogeneity, as exemplified by the divergence in acquired mutations

between multiple relapse samples in the two cases from the current study. Nevertheless, the observed temporal heterogeneity in DLBCL is relatively low compared to other lymphoproliferative diseases (LPD). In chronic lymphocytic leukemia (CLL) [39,40], follicular lymphoma (FL) [41,42], and mantle cell lymphoma (MCL) [43], relatively large temporal and spatial heterogeneity was observed. In contrast, mutational analysis of circulating cell free tumor DNA in classical Hodgkin lymphoma (HL) indicates a relatively constant mutation profile [44].

Secondly, mutations in several known targets of somatic hypermutation were detected as private mutations in relapse samples [35]. Although the most frequent base pair substitution (C:G > T:A) can arise as a consequence of canonical AID activity, it is also the most frequently observed base pair change caused by cyclophosphamide [21,36]. The type and impact of clonal evolution varies depending on the LPD, type of therapy, and involved genes and pathways. For example, CLL patients who relapse after ibrutinib acquire mutations in Bruton Tyrosine Kinase (BTK) or phospholipase C-γ2 (PLCγ2) [45], whereas ibrutinib refractory MCL patients acquire mutations within several pathways, including genes of the NF-kB pathway, the mTOR pathway, and epigenetic modifiers [46].

Third, a minority of mutations detected in the primary samples could not be detected in the relapse samples. The low MAF of these mutations is suggestive of sub clonal passenger mutations, a phenomenon that has also been observed in CLL [39]. This data is in line with the study by Morin et al, in which loss of mutations was observed infrequently in cases with VAF <0.2 [18]. Retention of the vast majority of mutations identified in the primary tumor samples is important for reliable assessment of minimal residual disease (MRD) in free circulating tumor DNA (ctDNA) using targeted approaches [38,47,48]. Focusing on sub clonal passenger mutations might lead to false negative MRD results. Using broad panel-based strategies reduces this potential risk and has revealed a success rate of 80–85%. Another main advantage of a broad-panel based ctDNA analysis is that it also allows detection of mutations not observed in the primary biopsy, thus at least partially overcoming the spatial heterogeneity of the tumor sample [38]. Interestingly, ctDNA based analysis of BTK revealed emerging mutations in two of three DLBCL patients receiving ibrutinib [38].

Fourth, through pathway analysis we observed enrichment for mutations in genes related to antigen presentation (HLA class I and II molecules (HLA locus), B2M, CALR) in relapse samples. Although the HLA locus is a highly polymorphic region and somatic mutation calling is prone to errors, the high frequency of mutations in antigen-presentation related genes observed is consistent with previous studies [49]. Approximately 75% of DLBCL exhibit a genetic basis for immune escape [21]. Recently, this type of immune editing has been linked with mutations in MYD88 and CD79B [20]. In DLBCL, mutations in genes involved in immune escape (e.g., B2M

and CD58) were associated with increased risk of relapse by selective pressure analysis [12]. Analysis of paired DLBCL samples showed somatic mutations, Indels, or chromosomal deletions targeting CD58 and B2M in five out of seven cases [18]. Mutations in these genes have been postulated as relapse-associated events [15]. Loss of HLA-molecules is frequently observed in DLBCL, and this might have implications for immunotherapy [50].

Fifth, we observed mutations in SOCS1 (5/6), PIM1 (5/6), and MYC (3/6) in multiple patients, with part of the mutations being relapse specific or enriched. Compared to the reported mutation frequencies in these genes in newly diagnosed DLBCL [51], the frequency seems to be enriched in R/R DLBCL in this study. Based on a mathematical approach, SOCS1 and PIM1 mutations were amongst the genes with the highest selective pressure estimates [12]. PIM1 mutations have been reported in 38% of R/R DLBCL ABC-type cases, and MYC mutations in 11% of R/R DLBCL cases [18]. In addition, an enrichment for SOCS1 and PIM1 mutations in matched tumor samples was not only observed in R/R DLBCL (4 out of 7 patients), but also in transformed FL (4 out of 7 patients), as well as in relapsed FL (2 out of 7 patients), further supporting the role of mutations in these genes in relation to relapse [38]. In the genomic model of Reddy et al., PIM1 and MYC mutations, but not SOCS1, were significantly correlated with decreased survival [19]. This is in line with our observation, which shows a relatively constant MAF for SOCS1 and an increased MAF for PIM1 mutations in two of five patients. The dynamics of PIM1 mutations should be taken into account when treating patients with BTK inhibition, since PIM1-stabilizing mutations affect upstream regulators and downstream targets of the NF-kB pathway, decreasing sensitivity of ABC-type DLBCL to BTK inhibition [52].

Finally, through pathway analysis we observed enrichment for possible therapy related genes related to trans membrane receptor tyrosine kinases (RTK) and genes involved in the JAK/STAT pathway. Gains of mutations in RTKs was previously observed in matched R/R DLBCL samples [15]. Both SOCS1 and PIM1 converge at the JAK/STAT signaling pathway. While SOCS1 inhibits JAK/STAT signaling, PIM1 expression is correlated with activation of STAT [51,53]. In addition to our observations, loss of the Interleukin 9 receptor (IL9R) locus was previously observed in three out of seven relapsed DLBCL cases [15], and STAT6 mutations were reported in 36% of R/R GCB-type DLBCL [18], both further implicating a role for the JAK-STAT pathway in relapse.
This study has several limitations. It clearly shows the challenge of obtaining relapse biopsies and good quality genomic DNA from FFPE tissues for WES. Of the 61 initially identified patients, we obtained reliable WES data of representative sequential tumor biopsies for only 6 patients (10%). In half of the relapse cases in our series a biopsy was omitted, and when available, again in half of the cases the biopsy was not sufficient for this type of analysis. Although histological confirmation of R/R DLBCL is advocated, it is often omitted for various reasons. In primary R-CHOP refractory patients hardly any re-biopsies are performed. This is reflected in the current

study, in which only half of the patients had histological confirmation. In an attempt to maximize the number of patients in this study, WES was performed on all but one excision biopsy, despite suboptimal DNA quantity and quality. Unfortunately, either primary or R/R samples of 8 out of 14 eligible cases failed library preparation, resulting in sufficient quality data for only 6 patients. Degradation of DNA during fixation and storage might lead to sequencing artifacts, including false positive C:G > T:A substitutions [54]. More than 90% of mutations detected by our pipeline in the primary samples could also be detected in the relapse sample, indicating that our filtering criteria successfully eliminated sequencing artifacts. To avoid such artifacts, it would be better to use fresh frozen samples. However, FFPE is the main mode of storing tissue samples [15,18]. We cannot completely rule out the presence of mutations in genes with low coverage in WES, as exemplified by FOXO1 [18].

An alternative approach to avoid fixation artifacts applied in more recent studies is the analysis of ctDNA. Initial studies showed the potential of using ctDNA to evaluate clonal evolution in LPDs [42,46,47]. Larger studies using ctDNA analysis could provide a comprehensive view on the mutational evolution of R/R DLBCL. Nonetheless, current ctDNA analysis encompasses preselected lists of target genes, while WES offers the best chance of discovering novel resistance-promoting mutations, especially when moving to more targeted therapy. Implementation of ctDNA analysis in clinical practice requires further standardization for purification and detection of mutations to achieve high sensitivity, especially for detection of MRD. Finally, our study is not powered to address the impact of individual mutations. Nevertheless, recurrent mutations with variable MAF were observed in three genes that all have been implicated with therapy resistance [18,19].

Conclusions

We show modest temporal heterogeneity between paired tumor samples with the acquisition of new mutations and enrichment of possible therapy resistant related genes. These mutational dynamics should be taken into account when setting up and analyzing biomarker-driven treatment strategies.

Tables

Table 1: Patient- and clinicopathological characteristics of the 6 patients evaluable for mutational analysis

Patient characteristics					Clinicopathological characteristics							Outcome	
ID	M/F	Age	Stage	IPI	Morphology	COO	IHC	FISH	Primary biopsy	Relapse biopsy 1	Relapse biopsy 2	EoT	PFS	OS
1	F	53	4	3	DLBCL	GCB	n.a.	Incon.	Jejunum	Lymph node	-	CR	7	101
2	M	45	2	2	DLBCL	ABC	n.a.	MYC-	Lymph node	Lymph node	-	CR	17	56 †
3	M	65	3	2	DLBCL	GCB	n.a.	MYC-	Soft tissue	Soft tissue	-	PR	7	14 †
4	F	57	3	1	DLBCL	ABC	CD20-	MYC-	Lymph node	Lymph node	-	PD	5	8 †
5	F	57	4	4	MYC+/ BCL6+#	Unclassified	CD5+	MYC+ BCL6+	Lymph node A	Lymph node B*	Lymph node C*	n.a.	14	36 †
6	M	79	1	2	DLBCL	GCB	n.a.	MYC-	Soft palate	Skin site A **	Skin site B **	CR	55	55 †

*Abbreviations: ABC, activated B-cell; COO, cell-of-origin as determined by the nCounter Lymph2Cx assay; CR, complete remission; DLBCL, diffuse large B-cell lymphoma; FISH, fluorescence in situ hybridization; GCB, germinal center B-cell; IHC, immunohistochemistry; IPI, international prognostic index; n.a., not applicable; OS, overall survival; PFS, progression free survival; PR, partial remission; EoT, end-of-treatment. # According to the WHO 2017 classification the case is classified as a High grade B-cell lymphoma with MYC and BCL6 rearrangement; * biopsies taken at the different time points; ** biopsies taken at the same time points; † patient deceased. Survival in months.*

Figures

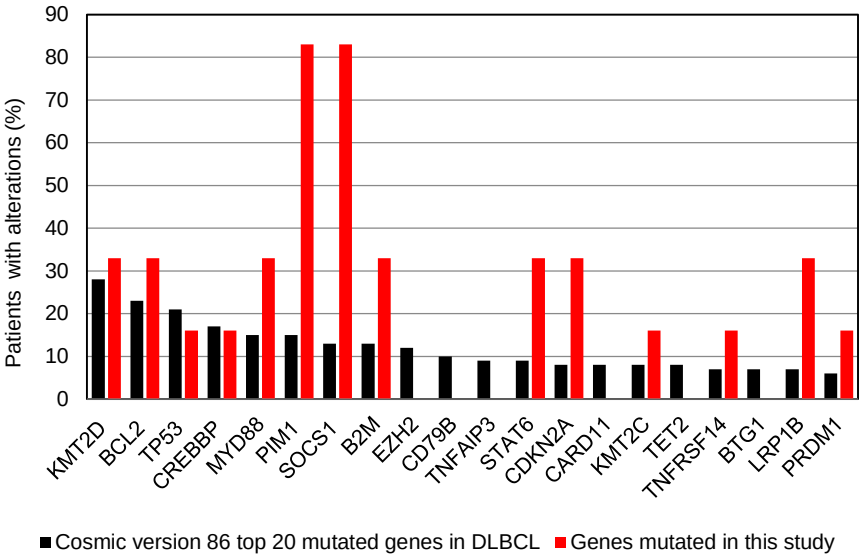


Figure 1. Frequency of mutations in the top-20 most commonly mutated genes in diffuse large B-cell lymphoma according to the Cosmic database version 86, and as observed in the 14 tumor samples analyzed in this study. Fourteen of the 20 genes were mutated in at least one of the 14 samples. SOC1 and PIM1 mutations were observed in 5 of 6 patients.

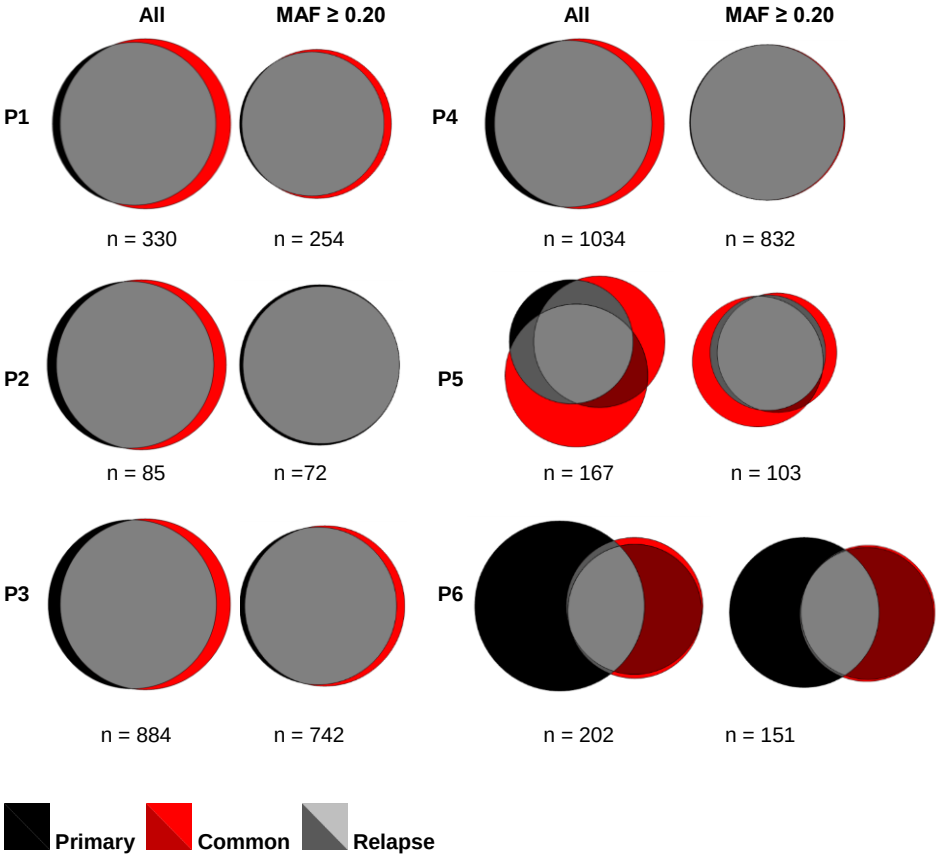


Figure 2. Venn diagrams showing for each individual patient (P1–6) the overlap in mutations between primary and paired relapse tumor samples. Left panels are Venn diagrams for all mutations (all), and right panels are Venn diagrams for mutations with a mutant allele frequency (MAF) ≥0.2. The total numbers of mutations per patient are depicted below each diagram. The size of the relapse diagram is proportional to the primary sample. In the patients with two biopsies at relapse (P5 and P6), the concordance between the novel mutations in the relapse samples was 45.3% and 89.2%, indicative of spatial heterogeneity.

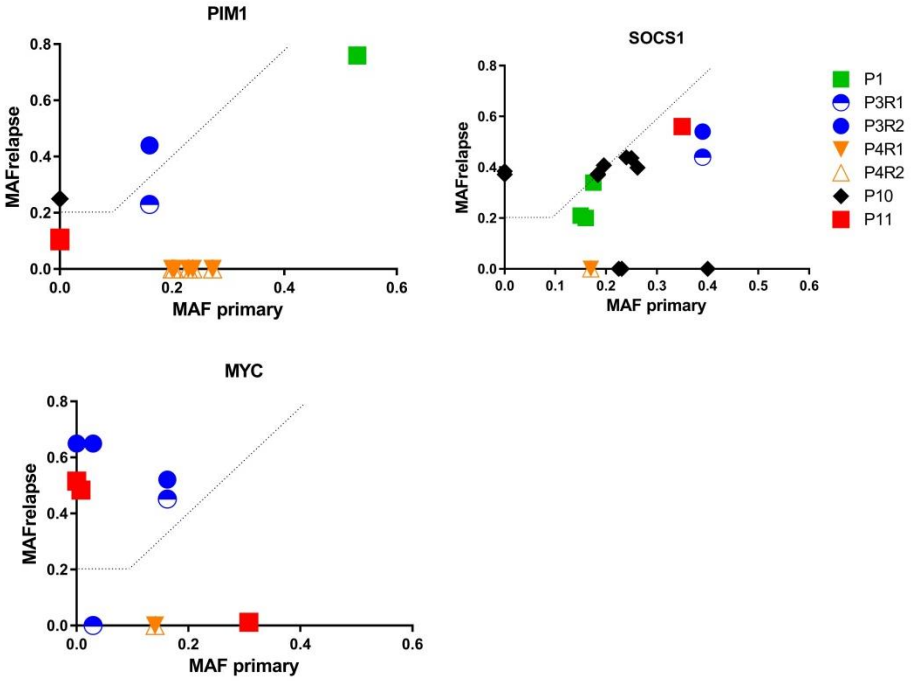
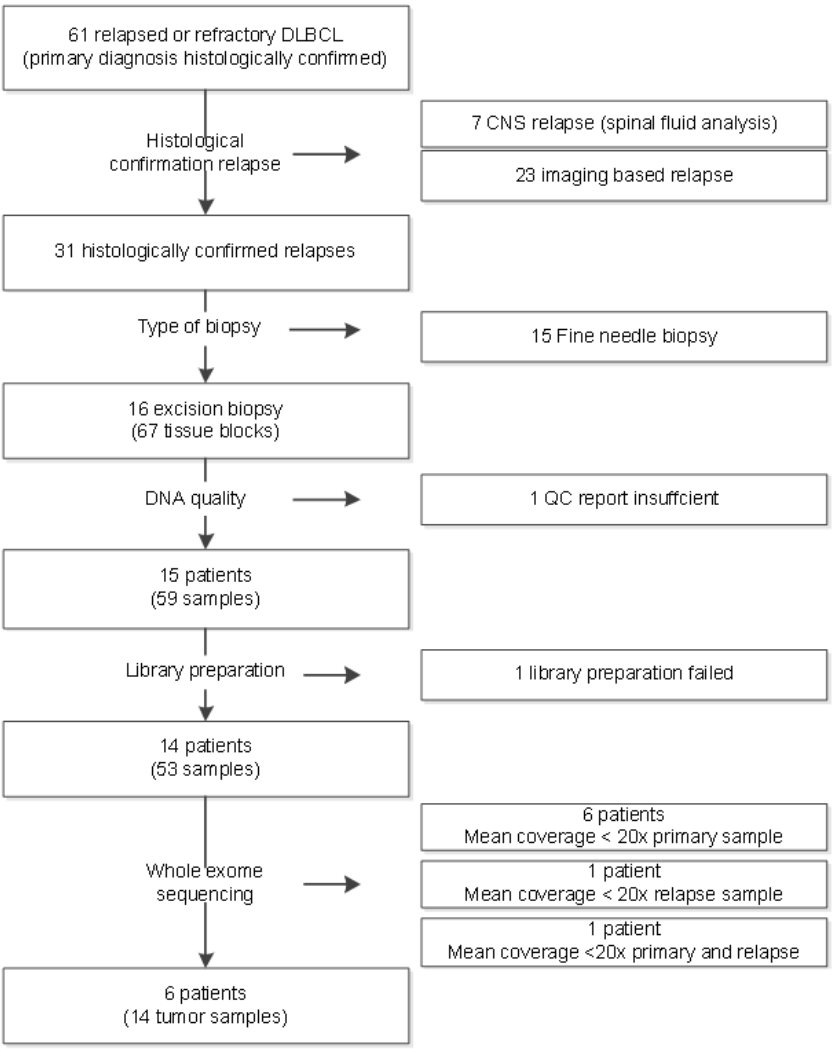
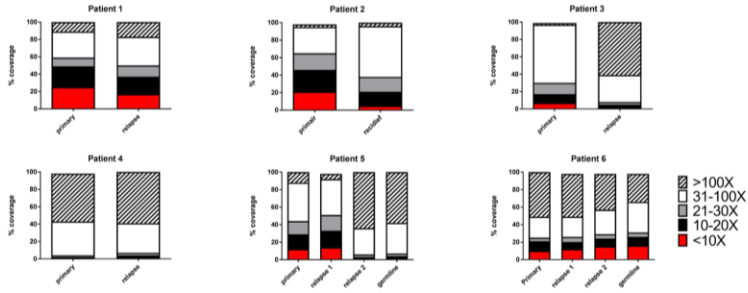


Figure 3. Graphical representation of mutant allele frequency (MAF) of (A) *SOCS1*, (B) *PIM1*, and (C) *MYC* in paired biopsies. Mutations above the dashed line are considered as possibly related to therapy resistance.

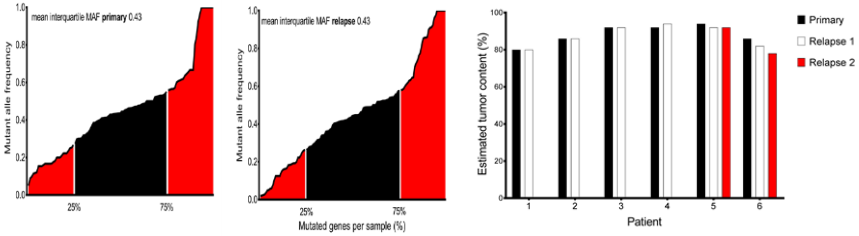
Supplementary



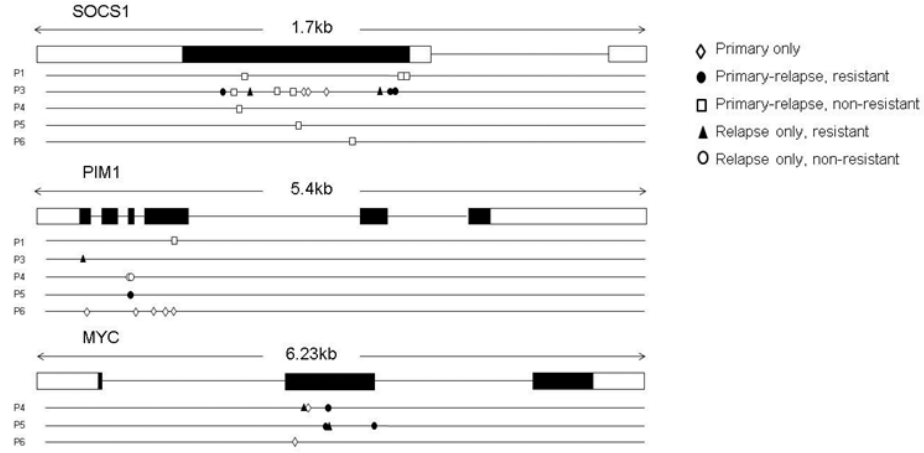
Supplementary figure 1: Schematic representation of patient and sample selection.



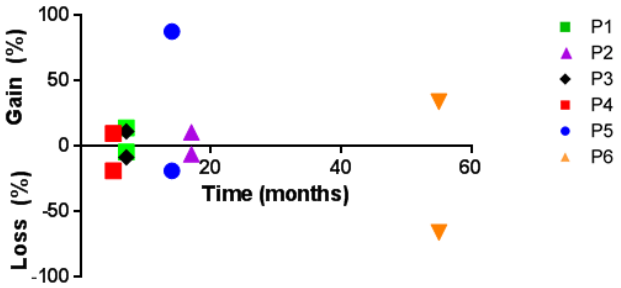
Supplementary figure 2: Percentage of the target region with indicated read depth (X) across the analyzed samples



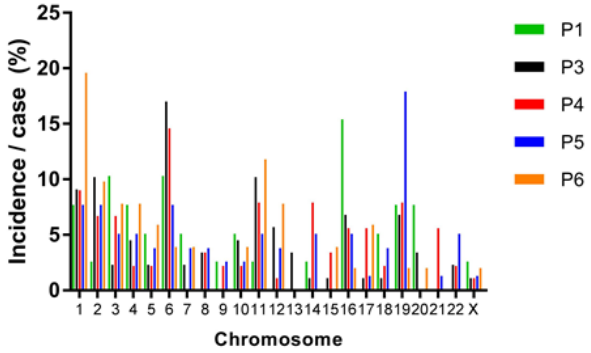
Supplementary figure 3: Estimated tumor cell percentages



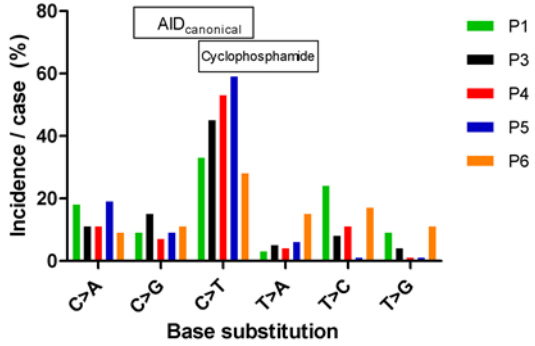
Supplementary figure 4: Schematic representation of location and type of mutations in SOCS1, PIM1, and MYC.



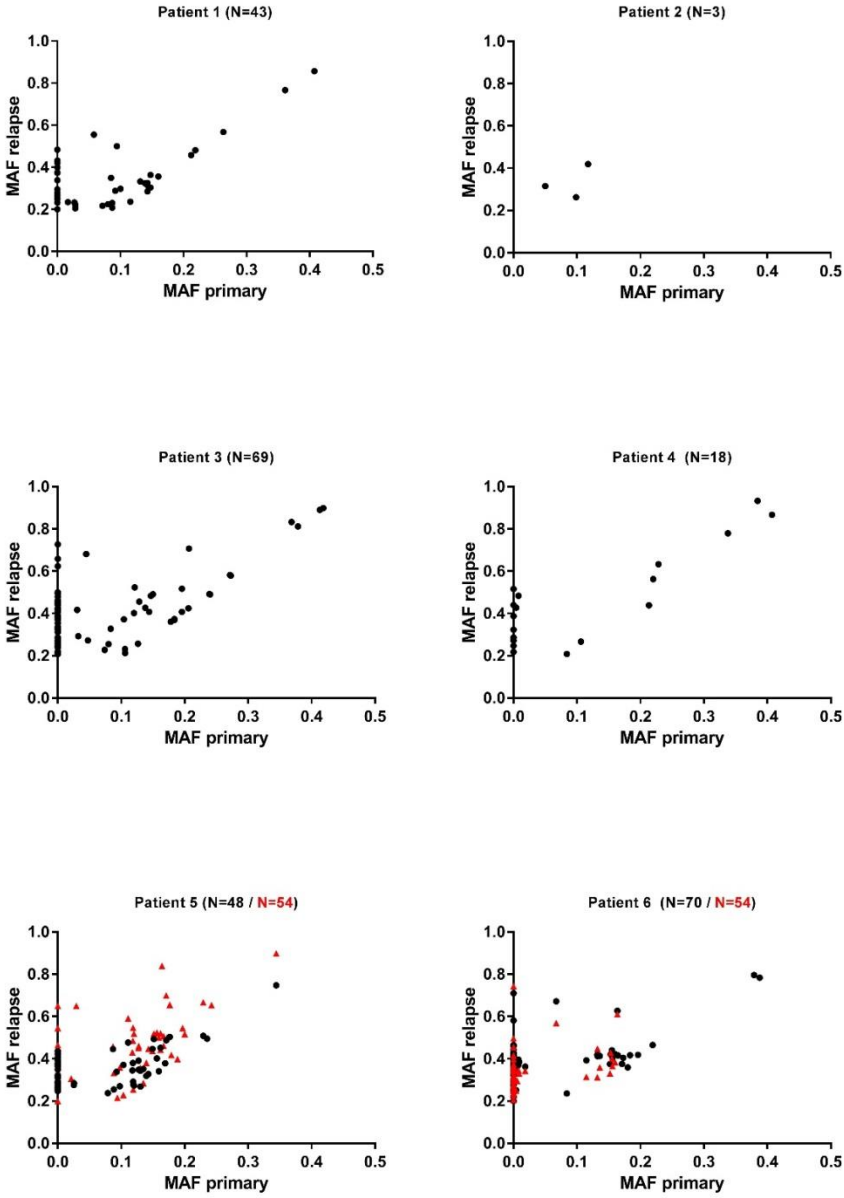
Supplementary figure 5: Loss and increase in mutational load as a variable over time did not show a significant correlation (*r*, 0.32; p, 0.71).



Supplementary figure 6: Distribution of mutations detected only in the relapse samples across the genome showed a random pattern



Supplementary figure 8: Prevalence of type of base substitutions across the relapse samples



Supplementary figure 7: Global view of mutant allele frequency (MAF) in primary and relapse samples of matched cases

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Chapter 8

Tumour necrosis as assessed with ^{18}F-FDG PET is a potential prognostic marker in diffuse large B-cell lymphoma independent of *MYC* rearrangements

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Abstract

Objectives: *MYC* gene rearrangements in diffuse large B-cell lymphomas (DLBCLs) result in high proliferation rates and are associated with a poor prognosis. Strong proliferation is associated with high metabolic demand and tumour necrosis. The aim of this study was to investigate differences in presence of necrosis and semiquantitative ^{18}F-FDG PET metrics between DLBCL cases with or without a *MYC* rearrangement. The prognostic impact of necrosis and semiquantitative ^{18}F-FDG PET parameters was investigated in an explorative survival analysis.

Methods: Fluorescence in situ hybridization analysis for *MYC* rearrangements, visual assesment, semi-quantitative analysis of ^{18}F-FDG PET scans and patient survival analysis were performed in 61 DLBCL patients, treated at a single referral hospital between 2008 and 2015.

Results: Of 61 tumours, 21 (34%) had a *MYC* rearrangement (*MYC*$^{+}$). *MYC* status was neither associated with the presence of necrosis on ^{18}F-FDG PET scans (necrosisPET; $P = 1.0$), nor with the investigated semiquantitative parameters maximum standard uptake value (SUV_{max}; $P = 0.43$), single highest SUV_{max} ($P = 0.49$), metabolic active tumour volume (MATV; $P = 0.68$) or total lesions glycolysis (TLG; $P = 0.62$). Multivariate patient survival analysis of the entire cohort showed necrosisPET as an independent prognostic marker for disease specific survival (DSS) (HR = 13.9; 95% CI: 3.0–65; $P = 0.001$).

Conclusions: *MYC* rearrangements in DLBCL have no influence on the visual parameter necrosisPET or the semi-quantiative parameters SUV_{max}, MATV and TLG. Irrespective of *MYC* rearrangements, necrosisPET is an independent, adverse prognostic factor for DSS.

Introduction

Diffuse large B-cell lymphoma (DLBCL) accounts for 35% of all B-cell non-Hodgkin lymphomas (B-NHL) [1]. Approximately 10-15% of DLBCL cases harbour a MYC gene rearrangement (MYC+), as assessed by fluorescence in situ hybridization (FISH) [2]. These lymphomas are characterized by a very high proliferation rate. Patients bearing a MYC+ lymphoma experience an aggressive clinical course and have a poor prognosis when treated with the standard regimen of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP) [3]. In 2017 the World Health Organization (WHO) established a new entity for MYC rearranged DLBCL, called 'high-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangements' [1, 4].
MYC is an oncogenic transcription factor regulating a vast array of cellular processes and pathways [5, 6]. Tumour cells overexpressing MYC, meet their high energy demands by increased glucose uptake, glycolysis, lactate production and amino acid consumption [7, 8]. However, unlike physiological tissues, cancer cells frequently have acquired resistance to apoptosis and cannot regulate their energy expenditure during metabolic stress, resulting in cell death via necrosis when nutrient supply is compromised [9–11].

In B-NHL patients 18F-fluorodeoxyglucose positron emission tomography (18F-FDG PET) scans are used for staging and response assessment [12]. Tumour necrosis can be assessed by visual inspection of 18F-FDG PET scans (necrosisPET) [13]. Necrosis can be observed in 14-20% of DLBCL cases and has been associated with an adverse prognosis [14, 15]. Semiquantitative assessment of 18F-FDG PET allows for relative comparison of parameters based on the spatial distribution and degree of 18F-FDG uptake and is currently being investigated as a tool for therapy monitoring and assessing prognosis in B-NHL [16–18]. Still, data on the prognostic value of the semiquantitative parameters maximum standardized uptake value (SUVmax) and metabolically active tumour volume (MATV) in DLBCL are conflicting [19–21]. MYC rearrangement, tumour necrosis (necrosisPET) and parameters derived from semiquantitative analysis of 18F-FDG PET are fundamentally linked to metabolism, yet the relationship between these factors remains unknown. We hypothesize, that the higher metabolic activity mediated by MYC rearrangements might result in a higher incidence of necrosisPET and increased semiquantitative parameters. The previously suggested prognostic impact of necrosisPET [15] and semiquantitative parameters [16–18] in DLBCL might be accredited to their potential association with MYC rearrangements.

Therefore, the aim of this study was to investigate differences in the presence of necrosisPET and semiquantitative 18F-FDG PET metrics between DLBCL cases with or without a MYC rearrangement. The prognostic impact of these factors was explored by means of survival analysis.

Material and methods

Study design

For this retrospective single centre study consecutive patients with newly diagnosed, histologically confirmed DLBCL between 2008 and 2015, were identified in the electronic healthcare database of the University Medical Center Groningen (UMCG), a reference centre for aggressive B-cell lymphomas. Cases of primary cutaneous DLBCL, primary central nervous system lymphoma, primary mediastinal B-cell lymphoma and immunodeficiency-associated lymphomas were excluded. The selection of cases for this study is summarized in Figure 1. Patients were stratified according to the National Comprehensive Cancer Network international prognostic index (NCCN-IPI) [22]. End of treatment response was assessed by 18F-FDG PET/CT scan. Tumour responses were classified according to Lugano criteria [12]. Follow-up was registered until early October 2017. According to Dutch regulations, no medical ethical committee approval was required for this retrospective, non-interventional study. A waiver was obtained from the medical ethics committee of the UMCG on November 13th 2018. The study utilized rest material from patients, the use of which is regulated under the code for good clinical practice in the Netherlands and does not require informed consent in accordance with Dutch regulations.

Pathology review

Pathology review was done using the 2008 WHO classification of haematopoietic and lymphoid tissues (AD) [23]. Histological scoring for necrosis (necrosisHist) was done by microscopic assessment of haematoxylin & eosin stained slides. Only microscopic areas with definite histopathological signs of necrosis (i.e. karyolysis) were scored as positive for necrosisHist.

MYC fluorescence in situ hybridization

For evaluation of a MYC rearrangement formalin-fixed paraffin-embedded tissue blocks of primary tumour samples were used. Interphase fluorescence in situ hybridization (FISH) was performed on 4-µm thick whole tissue sections, using Vysis break apart probes (Abbot technologies) and standard FISH protocols as previously described [24]. Researchers performing MYC FISH analyses were blinded for results from visual scoring, microscopic assessment of necrosis (necrosisHist) and clinical outcome.

18F-FDG PET imaging

All 18F-FDG PET scans were performed prior to therapy. Patients were allowed to continue all medication and fasted for at least 6 hours before whole-body (from skull vertex to mid-thigh level) three-dimensional PET images were acquired. This was done 60 minutes after intravenous administration of a standard dose of 3 MBq/kg (0.081 mCi/kg) bodyweight 18F-FDG on a Biograph mCT (Siemens Healthineers), according to the European Association of Nuclear

Medicine (EANM) procedure guidelines for tumour imaging with FDG PET/CT (version 2.0) [25]. Acquisition was performed in 7 bed positions of 2 minutes emission scan for patients 60-90 kg. Patient with body weight less than 60 kg and more than 90 kg body weight, were scanned with 1 minute and 3 minutes per bed position, respectively. Low dose transmission CT was used for attenuation correction. Low dose CT and 18F-FDG PET scans were automatically fused by use of three-dimensional fusion software (Siemens Healthineers) with manual fine adjustments. Raw data were reconstructed through ultra-high definition (Siemens Healthineers).

Computed tomography

Diagnostic CTs were acquired via integrated 18F-FDG PET/CT scans according to the European Association of Nuclear Medicine (EANM) procedure guidelines for tumour imaging with FDG PET/CT (version 2.0) [25]. Bulky disease was defined as any nodal lymphoma lesion > 10cm in coronal, axial or sagittal planes.

18F-FDG PET analysis

All 18F-FDG PET scans were visually assessed for the presence of tumour necrosis (necrosisPET) by an experienced reader (TCK), who was blinded to clinical, laboratory, biopsy, and follow-up findings, as previously described [15]. Areas within any nodal or extranodal 18F-FDG PET avid lymphomatous lesions that showed no 18F-FDG uptake were registered as having necrosisPET (Figure 2); no specific visual scale was used.

Semiquantitative analysis was performed using an in-house tool for quantitative 18F-FDG PET/CT analysis, as previously described [26–28]. This program automatically preselects lesions using a SUVmax threshold of 4 and a metabolic volume threshold of 2.5 ml. Unwanted preselected FDG-avid regions, such as bladder and brain, are removed by user interaction. Finally, remaining FDG-avid segmentations are processed using a background corrected 50% of SUV peak region growing method, as described by Frings et al [26], to obtain the final tumour segmentations. In case obvious lymphoma lesions were not selected (n=3), they were manually added after automatic tumour segmentation. From the final segmentation the metabolic active tumour volume (MATV in ml), total lesion glycolysis (TLG = MATV × SUVmean) and SUVs are derived for each lesion independently as well as summed over all lesions. Lesion selection and semiquantitative analysis was performed by MH under direct supervision of an experienced nuclear medicine physician (WN) and a nuclear physicist (RB). SUVmax was defined as the highest SUV per voxel within one lymphomatous lesion. In this paper SUVmax is reported as the mean of SUVmax across all lesions of an individual patient. SUVmax single highest was defined as the highest SUVmax of all lesions within an individual patient.

Statistical analysis

Comparison between continuous, non-normally distributed variables was estimated by Wilcoxon rank-sum test. Differences between two nominal variables were evaluated using Pearson's Chi

square or Fisher's exact test (for expected groups sizes ≤ 5). For exploratory survival analysis the primary endpoints were OS, progression free survival (PFS) and diseases specific survival (DSS). OS was defined as time from diagnosis until death (from any cause). PFS was defined as the time from diagnosis until death or relapse or progression [12]. DSS was defined as the time from diagnosis until death from DLBCL. Surviving patients were censored at the last date of follow-up. Survival curves were estimated according to the Kaplan-Meier method. Cox regression was used for univariate and multivariate survival analysis and results were reported as hazard ratio (HR), 95% confidence interval (CI) and P-value based on statistical Wald-test. A two-tailed P-value of less than 0.05 indicated statistical significance. All analyses were performed using R version 3.4.1 and R-studio version 1.0.153 software.

Results

Patient characteristics

Characteristics of the entire cohort (61 patients) are summarized in Table 1. A total of 21 patients (34%) had a DLBCL harbouring a *MYC* rearrangement. *MYC* rearrangement was observed in 11 patients (21.6%) primarily seen in the UMCG (n=51) and 10 patients (100%) referred from affiliated hospitals (n=10). *MYC* groups did not differ with regard to baseline characteristics (Table 1) except for serum LDH levels, which were higher in the *MYC*-positive group (P = 0.036) than cases without *MYC* rearrangement.

MYC status, necrosis and semiquantitative ^{18}F-FDG PET parameters

NecrosisPET was observed in 15 patients (25%). The relationships between *MYC* status and necrosisPET, necrosis^{Hist} and semiquantitative ^{18}F-FDG PET parameters are summarized in Table 2. *MYC*$^{+}$ cases did not differ from cases without *MYC* rearrangement with regards to necrosisPET (P= 1.0) or necrosis^{Hist} (P = 0.52). When the semiquantitative parameters SUV_{max}, SUV_{max} single highest, MATV and TLG, were studied, no difference between *MYC* groups was observed. There was no relation between the presence of necrosisPET and necrosis^{Hist} (P = 0.1; Supplementary Figure 1).

NecrosisPET and tumour volume

In 14 of 15 necrosisPET cases necrosis was observed in the largest lesion. In comparison, the largest individual lesion of cases without necrosisPET had a significantly lower MATV (P = 0.0006) and SUV_{max} (P = 0.02), irrespective of *MYC* status (Supplementary Figure 2). Bulky disease was observed in 24 patients (39%). Bulky disease was significantly correlated with necrosisPET (P = 0.005), but not with *MYC* status (P = 0.9) or necrosis^{Hist} (P = 0.8). Extranodal growth of lesions was not significantly correlated with the presence of necrosisPET (P = 0.26).

Survival analysis

The median follow-up was 34 months. At 5-years OS was 67% (95% CI: 54–83%), PFS was 65% (95% CI: 53–81%) and DSS was 81% (95% CI: 70– 93%) for the entire cohort. Of the seven deaths unrelated to lymphoma, two were caused by metastatic adenocarcinoma, two were due to cardiac failure, one was due to acute on chronic renal failure and there were two cases of sudden deaths in patients in complete remission of DLBCL.

Results of the univariate Cox regression analysis (HR, 95% CI and *P*-value) are shown in Table 3. Univariate analysis for OS identified *MYC*, NCCN-IPI and SUV_{max} single highest as associated factors. In univariate analysis for PFS only NCCN-IPI was associated with outcome. In univariate analysis for DSS *MYC*, NCCN-IPI, SUV_{max} single highest and necrosisPET were associated. Both SUV_{max} and SUV_{max} single highest showed negative beta-coefficients throughout the univariate survival analysis.

For multivariate analysis the parameters *MYC*, NCCN-IPI, necrosisPET and SUV_{max} single highest were used due to their prognostic impact on lymphoma-related deaths in univariate analysis (Table 4). NecrosisPET did not con-tribute to the prognostic model for OS and PFS. However, for DSS, necrosisPET had a large adverse prognostic impact and proved to be independent (HR = 13.9; 95% CI: 3.0–65; *P* = 0.001). Kaplan-Meier analysis for DSS showed no events during the 5 year follow-up period for patients who neither had *MYC* rearrangements, nor necrosisPET (n=30) (Figure 3).

Discussion

Based on the current investigation, there is no association of MYC rearrangements with the presence of tumour necrosis assessed by 18F-FDG PET or the semiquantitative 18F-FDG PET parameters SUVmax, SUVmax single highest, MATV and TLG, thus rejecting the hypothesis that metabolic changes induced by MYC rearrangements might increase the incidence of necrosisPET or alter the profile of semiquantitative parameters in DLBCL.

NecrosisPET was significantly associated with the MATV of the single largest tumour lesion. The SUVmax of the single largest necrosisPET lesion was significantly higher compared to the lesions without necrosisPET. Both of these observations support the notion of larger, more metabolically active tumours being more susceptible to necrosis, irrespective of MYC status.

Our analyses demonstrate that necrosisPET had a significant impact on DSS, thereby substantiating previous findings about the prognostic value of this visual marker [15]. The presented data show that presence of MYC rearrangement, in itself a powerful predictive factor, is not related to necrosisPET. This allows for integration of MYC status and necrosisPET into a prognostic model for DLBCL. When combined with MYC, NCCN-IPI and SUVmax single highest

in multivariate analysis, necrosisPET had the highest significance in predicting death due to lymphoma and a higher prognostic impact than NCCN-IPI, the currently most accurate prognostic index for DLBCL [22]. Thus, our results support the potential additive value of necrosisPET as an important biomarker for risk stratification in the clinical setting [14, 15].

The lack of a relationship between MYC rearrangements and semiquantitative 18F-FDG PET metrics might have several causes. First, proliferation in DLBCL could be independent of MYC rearrangement. This would only partially explain the lack of relationship, since the median proliferation index (Ki-67 staining) of MYC+ DLBCL is universally high (>90%) in contrast to the much broader range observed in MYC− DLBCL [29].

Second, overexpression of MYC via other mechanisms such as epigenetic pathways might explain increased glucose uptake in MYC FISH negative DLBCL. This is supported by studies showing high MYC protein expression in 19–40% of DLBCL cases [30–32]. Cottereau et al. previously reported a lack of relation between MYC protein expression and 18F-FDG PET parameters in DLBCL [19]. However, FISH analysis, which is considered the gold standard examination for MYC rearrangements [33–35], was not performed.

Third, high metabolic activity might be induced by alternative changes in metabolic drivers, such as mutations in PTEN (observed in approximately 15% of DLBCL) that lead to activation of the P13K/AKT/mTOR pathway [29, 36–38].

Intriguingly, univariate survival analysis indicated a protective effect for cases with SUVmax and SUVmax single highest measurements above the median. Studies on the prognostic impact of these variables are conflicting [20, 39–41]. Gallicchio et al. published results similar to ours, alluding to lymphomas with high metabolic activity being more responsive to chemotherapy [20]. In light of conflicting data on the prognostic value of semiquantitative 18F-FDG PET parameters [19–21, 42, 43], our results underline the need for larger, prospective studies with external validation cohorts [42].

This study has several limitations. First there is a referral bias with a high incidence of MYC+ cases (34%) in our dataset. The enrichment in our study can largely be explained by the fact that, as a reference centre, aggressive and MYC+ DLBCL cases (including suspected cases of Burkitt lymphoma which subsequently prove to be MYC+ DLBCL) are referred to our site. Second, the total number of cases with necrosisPET is small, which increases the risk of a sampling error. Nevertheless, the incidence of necrosisPET in our study is in line with previous studies [13–15]. Furthermore, patients were included irrespective of their comorbidities. Factors like differences in treatment regimen and non-cancer-related deaths might thus have a large impact on statistical

analysis. This is supported by the difference between DSS and OS. Despite its limitations, the prognostic potential of MYC status and NCCN-IPI was reproduced in this dataset making it a representative set of DLBCL cases. Larger prospective studies are warranted to validate the prognostic value of necrosisPET.

Conclusion

In this comprehensive analysis of MYC rearranged DLBCL we showed that a fundamental pathological change such as MYC rearrangement, which by itself has as significant impact on prognosis, has no influence on the presence of necrosisPET or semiquantitative 18F-FDG PET metrics. Explorative survival analysis suggests that the presence of necrosis determined by visual assessment of 18F-FDG PET scans is an independent predictor of disease specific survival in patients with DLBCL, regardless of MYC status.

Tables

Table 1. Demographics and baseline disease characteristics of patients with diffuse large B-cell lymphoma according to *MYC* status

			***MYC* status**				
	Total (n=61)		***MYC*$^{-}$(n=40)**		***MYC*$^{+}$ (n=21)**		***P*-value**
	No.	*%*	No.	*%*	No.	*%*	
Gender							
Male	36	*59.0*	24	*60.0*	12	*57.1*	1.0[a]
Female	25	*41.0*	16	*40.0*	9	*42.9*	
Age							
Median (range)	63 (26 - 91)		64 (26 - 91)		61 (30 - 79)		0.64[b]
Age ≤ 60y	24	*39.3*	14	*35.0*	10	*47.6*	0.5[a]
Age > 60y	37	*60.7*	26	*65.0*	11	*52.4*	
Stage							
I-II	22	*36.0*	15	*37.5*	7	*33.3*	0.97[a]
III-IV	39	*63.9*	25	*62.5*	14	*66.7*	
NCCN-IPI score							
0-3	30	*49.2*	22	*55.0*	8	*38.1*	0.32[a]
4-8	31	*50.8*	18	*45.0*	13	*61.9*	
Serum LDH							
Median (range)	282 (126 - 3037)		237 (126 - 1292)		381 (140 - 3037)		0.04[b]
Normal	29	*47.5*	22	*55.0*	7	*33.3*	0.18[a]
Elevated	32	*52.5*	18	*45.0*	14	*66.7*	
Treatment							
R-CHOP	56	*91.8*	37	*92.5*	19	*90.5*	0.36[c]
Intensive chemotherapy	3	*4.9*	1	*2.5*	2	*9.5*	
Palliative	2	*3.3*	2	*5.0*	0	*0*	

[a] *= Pearson's Chi-squared test with Yates' continuity correction*
[b] *= Wilcoxon rank sum test with continuity correction*
[c] *= Fischer's exact test for count data*

Table 2. Necrosis and semiquantitative ^{18}F-FDG PET parameters according to *MYC* status

			***MYC* status**				
	Total (n=61)		***MYC*$^{-}$ (n=40)**		***MYC*$^{+}$ (n=21)**		***P*-value**
	No.	***%***	**No.**	***%***	**No.**	***%***	
NecrosisPET							
Absent	46	*75.4*	30	*75.0*	16	*76.2*	1.0[c]
Present	15	*24.6*	10	*25.0*	5	*23.8*	
Necrosis^{Hist}							
Absent	42	*68.9*	28	*70.0*	14	*66.7*	0.52[c]
Present	16	*26.2*	11	*27.5*	5	*23.8*	
Not available	3	*4.9*	1	*2.5*	2	*9.5*	
SUV_{max}							
Median (range)	13.0 (3.0 - 38.4)		13.1 (3.0 - 33.9)		10.4 (5.8 - 38.4)		0.43[b]
SUV_{max} single highest							
Median (range)	18.8 (3.8 - 45.8)		19.7 (3.8 - 39.0)		14.2 (5.8 - 45.8)		0.49[b]
MATV							
Median (range)	154.7 (1 - 3774)		156.0 (1 - 2800)		154.7 (7 - 3774)		0.68[b]
TLG							
Median (range)	1387.4 (3 - 29462)		1632.8 (3 - 29462)		1147.1 (47 - 20065)		0.62[b]

[b] = *Wilcoxon rank sum test*
[c] = *Fisher's exact test for count data*

Table 3. Univariate analysis of patient characteristics and semiquantitative ^{18}F-FDG PET parameters on overall survival, progression free survival and disease specific survival

	HR OS	95%CI	*P*-value	HR PFS	95%CI	*P*-value	HR DSS	95%CI	*P*-value
MYC									
MYC -	Ref.			Ref.			Ref.		
MYC +	2.9	1.1 - 7.4	0.025*	2.3	0.97 - 5.7	0.058	6.3	1.7 - 24	0.007**
NCCN-IPI									
0-3	Ref.			Ref.			Ref.		
4-8	3.0	1.0 - 8.3	0.04*	3.6	1.3 - 10	0.013*	10.7	1.4 - 84	0.024*
Necrosis^{PET}									
Absent	Ref.			Ref.			Ref.		
Present	1.7	0.6 - 4.5	0.3	1.8	0.7 - 4.6	0.2	3.9	1.2 - 13	0.025*
SUV_{max}									
< Median	Ref.			Ref.			Ref.		
≥ Median	0.4	0.1 - 1.1	0.08	0.4	0.2 - 1.1	0.08	0.2	0.05 - 1.1	0.06
SUV_{max} single highest									
< Median	Ref.			Ref.			Ref.		
≥ Median	0.3	0.09 - 0.9	0.026*	0.4	0.2 - 1.1	0.07	0.1	0.01 - 0.8	0.028*
MATV									
< Median	Ref.			Ref.			Ref.		
≥ Median	1.1	0.4 - 2.7	0.9	1.3	0.5 - 3.1	0.59	2.8	0.7 - 10.6	0.14
TLG									
< Median	Ref.			Ref.			Ref.		
≥ Median	0.6	0.2 - 1.6	0.31	0.8	0.3 - 1.9	0.57	1.1	0.3 - 3.8	0.84

Table 4. Multivariate analysis of patient characteristics overall survival, progression free survival and disease specific survival.

	Hazard Ratio OS	95% CI	*P*-value[#]	*P*-value Model
MYC				
MYC-negative	Reference			
MYC-positive	3.1	1.1 – 8.7	0.029*	
NCCN-IPI				
0-3	Reference			
4-8	2.4	0.8 – 6.9	0.116	0.004
NecrosisPET				
Absent	Reference			
Present	2.6	0.9 – 7.7	0.079	
SUV_{max} single highest				
< Median	Reference			
≥ Median	0.3	0.1 – 0.9	0.027*	
	Hazard Ratio PFS			
MYC				
MYC-negative	Reference			
MYC-positive	2.4	0.9 – 6.3	0.07	
NCCN-IPI				
0-3	Reference			
4-8	3.2	1.1 – 9.0	0.028*	0.005
NecrosisPET				
Absent	Reference			
Present	2.6	1.0 – 7.0	0.06	
SUV_{max} single highest				
< Median	Reference			
≥ Median	0.4	0.2 – 1.1	0.08	
	Hazard Ratio DSS			
MYC				
MYC-negative	Reference			
MYC-positive	14.6	2.6 – 82	0.002**	
NCCN-IPI				
0-3	Reference			
4-8	6.5	0.6 - 66	0.113	0.0007
NecrosisPET				
Absent	Reference			
Present	13.3	2.8 – 63	0.001**	
SUV_{max} single highest				
< Median	Reference			
≥ Median	0.12	0.01 – 1.2	0.075	

[#] Wald-test

Figures

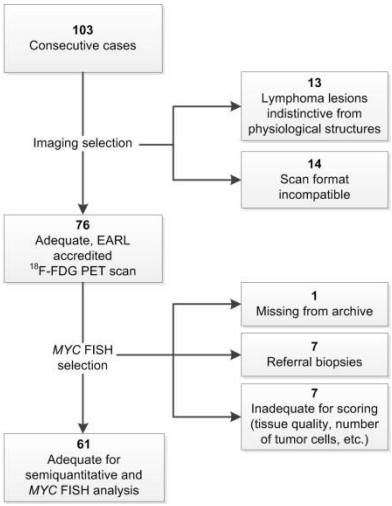


Figure 1. Flow-chart of case selection.

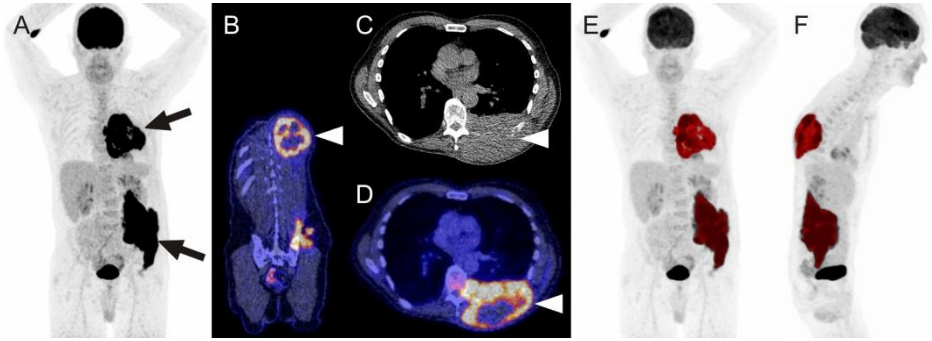


Figure 2. Visual assessment of necrosis and semi-quantitative ^{18}F-FDG PET review process. (A) 65-year-old man with diffuse large B-cell lymphoma (DLBCL) and tumour masses in the left dorsal chest wall and left pelvis, as shown on the coronal maximum intensity projection (MIP) ^{18}F-FDG PET image (arrows). Coronal fused ^{18}F-FDG PET /CT (B), axial CT (C) and axial fused ^{18}F-FDG PET/CT (D) show the tumour mass with photopenic areas (arrow heads), in keeping with tumour necrosis. Coronal and sagittal MIP ^{18}F-FDG PET images (E and F) show tumour segmentation (marked in red colour) for the calculation of metabolically active tumour volume (MATV), total lesion glycolysis (TLG), maximum standard uptake value (SUV_{max}), and single highest SUV_{max}.

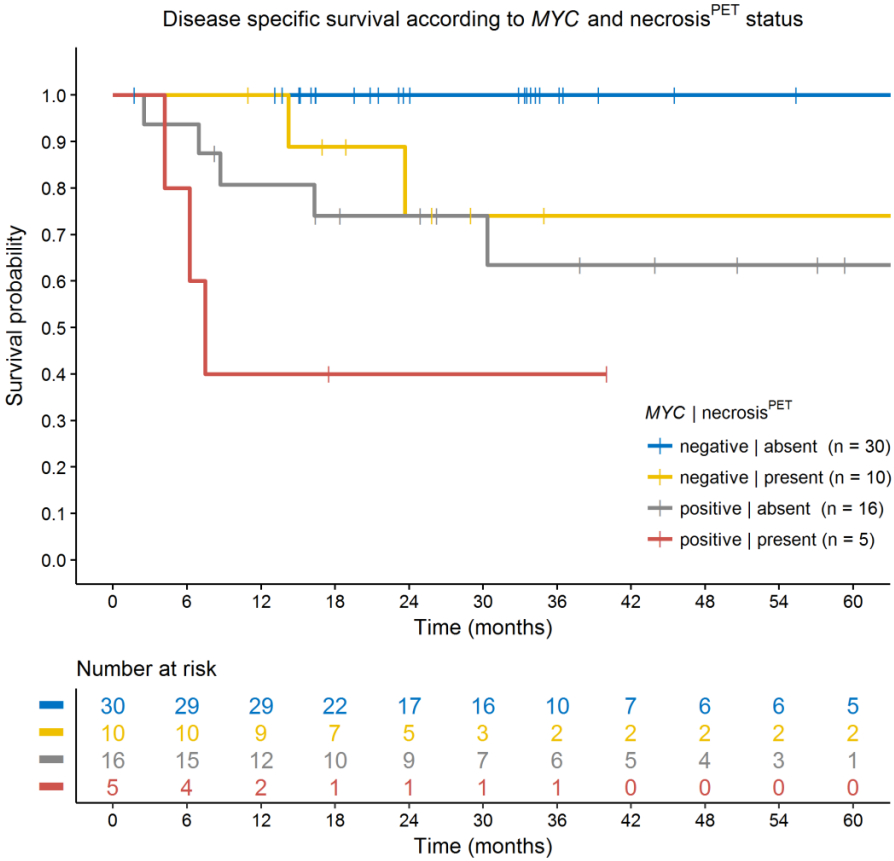
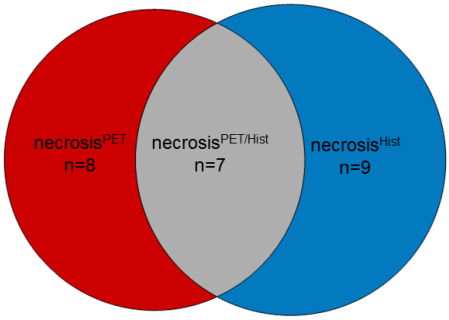
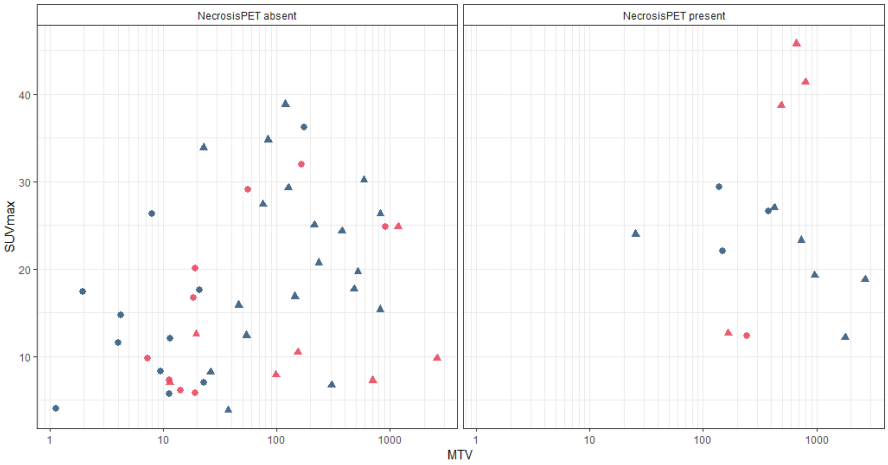


Figure 3. Kaplan-Meier curve showing disease specific survival according to combined analysis with *MYC* rearrangement status and necrosisPET (log-rank test, $P = 0.00022$). No events were observed in patients without *MYC* rearrangement and who had no necrosisPET.

Supplementary



Supplementary Figure 1. Venn diagram showing the overlap between necrosisPET and necrosis^{Hist} scoring indicating poor concordance.



Supplementary Figure 2. Distribution of metabolically active tumour volume (MATV) and maximum standard uptake value (SUV_{max}) of single largest tumour lesions in relation to the absence or presence of necrosisPET. MYC-negative, non-confluent MYC-positive, non-confluent MYC-negative, confluent MYC-positive, confluent. MATV (P = 0.0006) and SUV_{max} (P = 0.02) were significantly higher in patients with necrosisPET. MATV and SUV_{max} showed no significant difference between *MYC* groups.

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Chapter 9

False positive spinal cord uptake on fluorodeoxyglucose positron-emission tomography following treatment of lymphoma

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British Journal of Hematology, September 2012; 159: 497

Case report

A 19-year-old woman with no previous medical history was diagnosed with a stage IV primary mediastinal large B-cell lymphoma with a bulky mass and an International Prognostic Index score of 3 (figure 1A). After 6 cycles of R-CHOP14 (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone at 14 d intervals) a combined ^{18}F-fluorodeoxyglucose positron-emission tomography (^{18}F-FDG-PET) and computed tomography (CT) scan was performed (Siemens biograph mCT with 64 slices, 135 MBq ^{18}F-FDG with at least 6 h fasting prior to intravenous injection). The scan showed a partial remission with persisting ^{18}F-FDG PET-positive mediastinal lymphadenopathy. She received 30 Gy radiation and a boost up to 40 Gy to the ^{18}F-FDG PET-positive lesions. Response evaluation after treatment showed intense ^{18}F-FDG uptake in the spinal cord at the level of the eleventh and twelfth thoracic vertebrae (Figure 1B). She had no neurological symptoms.

The role of ^{18}F-FDG PET in staging and evaluation of remission in lymphomas is well established. The incidence of central nervous system (CNS) recurrence is reported to be 2–5%. In this case, the end-of-treatment ^{18}F-FDG PET showed abnormal spinal ^{18}F-FDG uptake and CNS progression of the lymphoma was considered. Magnetic resonance imaging of the spinal cord showed no lesions. The ^{18}F-FDG uptake in the spinal cord was therefore considered to be false positive. It appears that this physiological phenomenon is encountered in up to half of cancer patients and is seen more often in winter.[1] Increased ^{18}F-FDG uptake is primarily seen at the level of the cervical vertebrae, the eleventh and twelfth dorsal vertebrae, and the first lumbar vertebrae. The CNS is highly dependent on glucose and accounts for 50% of glucose metabolism. During fasting and stress conditions, such as during the ^{18}F-FDG PET-CT procedure, there is a physiological increase in glucose uptake in the CNS to maintain a steady glucose state. This mechanism probably accounts for the increased ^{18}F-FDG uptake observed in the spinal cord of our patient.

This case illustrates the potential pitfalls of normal physiological ^{18}F-FDG uptake in the spinal cord, which is frequently encountered in cancer patients. Awareness of this pitfall can avoid additional diagnostic procedures

Figures

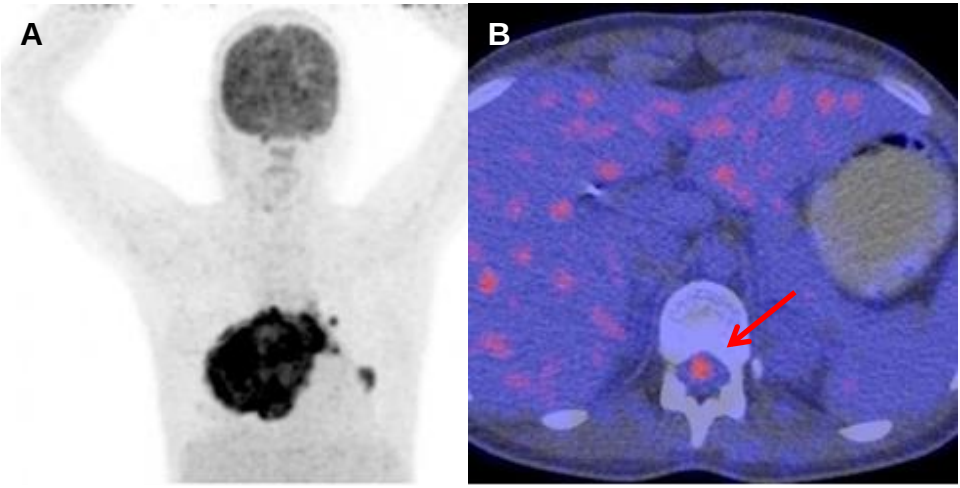

Figure 1: (**A**)^{18}F-FDG-PET scan before treatment showing a large mediastinal mass (**B**) ^{18}F-FDG-PET with false positive spinal FDG-uptake after treatment (red arrow)

References

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Chapter 10

Treatment of initial parenchymal central nervous system involvement in systemic aggressive B-cell lymphoma

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Abstract

Central nervous system (CNS) involvement in systemic B-cell non-Hodgkin lymphoma (B-NHL) at diagnosis (sysCNS) is rare. We investigated the outcome of 21 patients with sysCNS, most commonly diffuse large B-cell lymphoma, treated with high dose methotrexate (HD-MTX) and R-CHOP. The median number of cycles of HD-MTX and R-CHOP was 4 (range 1–8) and 6 (range 0–8), respectively. Consolidative whole brain radiotherapy (WBRT) was given to 33% (7/21) patients. With a median follow-up of 44 months the 3-year progression free survival (PFS) and overall survival (OS) were 45% (95%CI 34–56%) and 49% (95%CI 38–60%), respectively. Over 90% of patients had an unfavorable international prognostic index score, reflected by treatment-related mortality of 19% (4/21) and relapse-related mortality of 28% (6/21). The outcome of these patients was, however, unexpectedly good when compared to secondary CNS relapses. Prospective studies are needed to define the optimal treatment for patients with sysCNS, but its rarity might be challenging.

Introduction

The risk of central nervous system (CNS) dissemination of aggressive B-cell lymphoma (B-NHL) is 5% [1]. High international prognostic index (IPI) score, kidney or adrenal gland involvement and diffuse large B-cell lymphoma (DLBCL) with dual expression of MYC and BCL2 by immunohistochemistry convey a high risk of CNS relapse [2,3]. In the majority, CNS involvement occurs during progression of the disease. Rarely, CNS dissemination is already present at initial diagnosis. Unlike the general favorable outcome of patients with primary CNS lymphoma (PCNSL) when treated with high dose methotrexate (HD-MTX), cytarabine and thiothepa [4–7], CNS relapse of aggressive B-NHL conveys a dismal prognosis [8,9]. Salvage regimens with relapsed PCNSL incorporating thiothepa and high dose chemotherapy followed by autologous stem cell transplantation (ASCT) showed an improved overall survival (OS) for these patients if initially responsive and subsequently eligible for intensive treatment [10,11].

It is unknown if the prognosis of concomitant systemic and parenchymal CNS B-NHL at diagnosis (sysCNS B-NHL) is different from secondary CNS lymphoma in the relapse setting. Given its rarity, data on treatment for sysCNS B-NHL are sparse and there is no established standard of care. Recent data from two studies suggest a beneficial effect of intensive chemotherapy followed by ASCT [12,13]. In both studies, ASCT was only offered to patients in complete remission (CR) (31%–50%). Furthermore, in one of the studies, anthracyclines – highly effective in systemic B-NHL – were only a minor part of the induction regimen [13]. Outcome of non-transplant strategies in sysCNS B-NHL was only explored in small series [14,15].

We observed an excellent response in a patient with sysCNS initially treated as if primary CNS with the European Organisation for Research and Treatment of Cancer (EORTC) regimen MBVP (methotrexate, etoposide, carmustine and prednisolone) [5]. In this patient, not only the CNS component responded well, but also systemic involvement responded at the same time. Consequently, we continued after MBVP with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP), and applied this regimen to the next series of patients. Simultaneously, another University Medical Center in the Netherlands combined R-CHOP alternating with HD-MTX, based upon the same observations.

Therefore, we retrospectively investigated the outcome of patients with sysCNS B-NHL treated in both centers with a combination of drugs effective for CNS and systemic involvement. None of these patients underwent ASCT.

Methods

Study design and patient identification

Clinical data on patients with sysCNS B-NHL were retrieved from the electronical databases of two referral medical centres in the Netherlands. All patients had newly diagnosed, histologically proven aggressive B-cell lymphoma. In case of transformed lymphoma, patients had not received prior therapy. CNS involvement was determined either by biopsy, magnetic resonance imaging (MRI) and/or CSF examination. Patients had been treated between 2000 and 2015. Patients should have received at least one cycle of HD-MTX. Both regimens start with a cycle of HD-MTX. The pathological assessment was performed by experienced hematopathologists. Since patients were treated according to best practice no medical ethical committee approval was required.

Initial assessment

Staging consisted of computed tomography scan of neck, chest, abdomen and pelvis, bone marrow biopsy, cerebrospinal fluid (CSF) examination with four-color flow cytometry (FCM), MRI brain, and further investigations if clinically indicated. A fluorodeoxyglucose (^{18}F-FDG) positron emission tomography scan was available for all patients treated at one of the medical centres. Systemic B-NHL was diagnosed by lymph node or tissue biopsies in all cases. CNS involvement was diagnosed either by brain biopsy or CSF flow cytometry in 58% (12/21) of patients. In 42% (9/21) neurological symptoms and parenchymal abnormalities on the MRI scan were considered sufficient for the diagnosis of CNS involvement. In 10% (2/21) CNS involvement presented as neurolymphomatosis. In all patients the international prognostic index (IPI) was documented.

Treatment

Patients were treated according to one of two regimens. At the University Medical Center Groningen a sequential regimen was adopted. Four cycles of MTX at a dose of 3 g/m^2 were given as part of the MBVP regimen, which also includes carmustine (100 mg/m^2 at day 4), teniposide (100 mg/m^2 at day 2 and 3) and prednisolone (60 mg/m^2 at day 1–5) [5]. Interim response evaluation was performed after completion of HD-MTX. Patients with a partial CNS remission received whole brain radiotherapy (WBRT) at this point. Patients subsequently continued for 6 cycles of R-CHOP after which end-of-treatment evaluation took place. R-CHOP was given as a combination of rituximab (375 mg/m^2 at day 1), cyclophosphamide (750 mg/m^2 at day 1), doxorubicin (50 mg/m^2 at day 1), vincristine (1.4 mg/m^2 at day 1 up to a maximal dose of 2 mg) and prednisolone (100 mg at day 1–5). At the Erasmus Medical Centre Rotterdam an alternating regimen was adopted, which consisted of 6 alternating cycles of HD-MTX at a dose of 3 g/m^2 and R-CHOP. Interim evaluation was performed after 3 cycles and end-of-treatment evaluation after completion of therapy. Patients with a partial remission (PR) of the CNS involvement received WBRT. In both regimens patients with leptomeningeal involvement received intrathecally

methotrexate and/or cytarabine until complete clearance of the CSF as assessed by FCM. Furthermore, 4 patients in the alternating regimen received i.t. therapy without a positive CSF because of local practice.

Response assessment

Response was evaluated according to the 1999 consensus criteria for systemic NHL and the consensus criteria for PCNSL [16,17]. Tumor response was classified as CR, PR, stable disease (SD), or progressive disease (PD). Only patients in complete remission at CNS and systemic sites at the end of treatment were reported as a CR. Treatment related mortality (TRM) was defined as death during or ≤3 months after treatment. Follow-up was completed until February 2016.

Statistical analysis

Duration of follow-up was calculated for all patients alive. The primary endpoints were OS and progression free survival (PFS). OS was defined as time from diagnosis until death (from any cause); PFS as the time from diagnosis until relapse, progression or death (from any cause). Survival curves were estimated according to the Kaplan–Meier method. Between-group differences in OS and TTP were evaluated using the log-rank test. All categorical variables were expressed as counts and percentages. Where applicable, differences between groups were evaluated by chi-square for binary variables and independent T-tests for continuous variables. A two-tailed *p*-value of less than 0.05 indicated statistical significance. All analyses were performed using IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY).

Results

Clinical characteristics

Between 2000 and 2015 21 sysCNS B-NHL patients were treated. Demographic data from the patients in the two treatment regimens are presented in Table 1. Diffuse large B-cell lymphoma composed the major histological B-NHL entity (66%). The median age was 54 years (range 19–71 years) with a slight female preponderance. Two-third of patients had a WHO performance score ≥2 and 91% of patients had an intermediate or high IPI-score. A large number of patients had lymphoma infiltration of organs known to convey a high risk of CNS relapse, like testicles (44% of patients at risk), bone (38%), kidney (19%) and breast (17% of patients at risk). Besides parenchymal CNS, concomitant CSF involvement was present in 43% of patients.

Treatment

Thirteen patients (62%) completed therapy with only minor deviations. Two patients received less than 6 R-CHOP cycles. One patient had both a reduction in the number of MTX and R-CHOP cycles. Data on treatment intensity between the two treatment regimens are presented in Table 2. Detailed information on individual treatment and response assessment is shown in Supplementary Figure 1. The median number of HD-MTX cycles was 4 (range 1–8). Thirteen patients (62%) received concomitant intrathecal MTX. The median number of R-CHOP cycles was 6 (range 0–8). WBRT was given to 7 patients (33%), all because of persistent abnormalities on brain MRI. One patient received local radiotherapy on an extracranial site because of PR at the end of treatment. Besides the additional VP16 and BCNU in patients treated with MBVP, there was no significant difference between the two regimens

Follow-up, response and survival

The median duration of follow-up of patients was 44 months (range 5–104). The overall response rate (ORR) at the end of treatment was 62%: CR 57% ($n = 12$) and PR 5% ($n = 1$). PD was noted in 19% ($n = 4$), whereas early TRM due to infectious complications was observed in 19% ($n = 4$). All infectious complications occurred during neutropenia. Two patients died as a consequence of an aspiration pneumonia due to neurogenic dysphagia. One patient developed a complicated clostridium difficile infection after prolonged treatment with ciprofloxacin. One patient died of a complicated meningitis (*Klebsiella pneumoniae*), which was not related to a previous lumbar puncture. One patient died 6 months after treatment as a result of cognitive deterioration.

Three-year PFS and OS for the entire cohort were 45% (95%CI 34–56%) and 49% (95%CI 38–60%), respectively (Figure 1 A,B). No significant differences in PFS and OS were found between both regimens (HR OS 1.25, 95%CI 0.38–4.2; p 0.72). Although statistical analysis showed no significant differences in outcome between the various variables due to the small number of patients, all patients ($n = 3$) with IPI-score 0–1 were alive at follow-up, whereas all patients ($n = 4$) with IPI-score 4–5 died within 2 years after diagnosis.

The combined incidence of refractory and relapsed sysCNS was 28% (6 of 21 patients). The relapse rate in patients achieving a CR was only 8% (1 of 12 patients), which is reflected by the plateau after 12 months in the PFS and OS curves (Figure 1(A,B)). Patients in CR/PR had a 3-year PFS and OS of 74% and 83%, respectively (Supplementary Figure 2 A,B). The patient who relapsed did so within a year in the CNS. She subsequently received WBRT but died soon afterwards. The one patient in PR progressed after 8 months with both systemic and CNS involvement. He received no further therapy and died shortly thereafter. The four patients with PD all died within 6 months. The site of progression was systemic-only in two patients, CNS-only in one patient and concomitant systemic and CNS in one patient.

Discussion

By combining the results from two medical referral centres in the Netherlands, we performed the second largest retrospective study in patients with sysCNS B-NHL. Findings from this study indicate that non-transplant regimens of HD-MTX and R-CHOP can be an effective treatment for sysCNS B-NHL. With a 3-year OS of 49% the outcome in our cohort is comparable to the more intensive regimen including ASCT, which is available to only a selected group of patients (5-year OS 41%) [13]. In one of the three studies with non-transplant regimens 4 out of 6 (67%) patients achieved a CR when treated with HD-MTX and R-CHOP. In a second study with HD-MTX, rituximab, cytarabine and idarubicin (R-IDARAM) 4 out of 10 (40%) patients achieved a CR. Median OS of both studies was 25.1 months and more than 30 months, respectively [14,18]. In a pilot study with dose-adjusted EPOCH (etoposide, cyclophosphamide, doxorubicin, vincristine, prednisone) plus rituximab 8 out of 8 patients achieved a CR [15]. Four patients underwent a consolidative autologous stem cell transplantation. With a median follow up of 11 months all patients were alive.

In most of our patients systemic involvement included one or more organs known to convey a high risk of CNS involvement in systemic B-NHL [1]. These findings suggest an early dissemination of a systemic B-NHL, rather than a systemic manifestation of a PCNSL. This might be important, since PCSNL has several pathological features that are only infrequently present in systemic DLBCL, such as a high incidence of mutations in *CD79B* and *MYD88*, loss of human leukocyte adhesion (HLA) molecules and frequent expression of the programed death ligand 1 [19–21]. Despite its similarity with systemic B-NHL, the OS of sysCNS B-NHL treated with HD-MTX and R-CHOP was better than expected and comparable to that of PCNSL and certainly not as bad as secondary relapsed CNS B-NHL treated with HD-MTX and cytarabine (Supplementary Table 1) [4–6,8].

The TRM in our cohort of 19% was high, and reflects the very unfavorable risk profile of our patients with 67% having a WHO performance of 3–4 and 14% being older than 65-years. Given the small numbers it cannot be answered whether the concomitant systemic involvement or additional R-CHOP played a role.

Despite the limited number of relapses, two observations were interesting: firstly, all but one patient who relapsed did so less than 6 months after the end of treatment. All other patients who failed did so during the early phase of treatment; secondly, in refractory and relapsing patients the majority had systemic progression. The incidence of refractory/early progressing patients in our cohort (24%) was similar to that of more intensive regimens (35%) [13]. This means that progressive patients have a dismal outcome and are unlikely to be salvaged. It is interesting to speculate whether drugs such as bendamustine, ibrutinib, checkpoint inhibitors and lenalidomide would be effective in this setting, since these drugs generally have a favorable toxicity profile, known activity in relapsed systemic B-NHL and CNS penetration [22–26].

Notably, patients who obtain a CR have an excellent outcome and probably will not benefit from additional high dose therapy, especially those patients with a low IPI at start. This challenges the role of consolidation as suggested by the French lymphoma group LYSA, that showed a positive impact of high-dose therapy followed by autologous stem cell transplantation on 3-year OS and PFS [12]. However, in our series 7 of 21 patients (33%) received consolidative radiotherapy. The indirect answer if ASCT can replace WBRT will have to come from the analysis of the IELSG32 trial, where patients with a chemosensitive PCNSL were randomized in a second randomization to WBRT or ASCT [7]. But even then we have to be cautious to extrapolate these findings to sysCNS, since PCSNL has a different biology.

Our study obviously has limitations. Firstly, its retrospective nature has inherent known and unknown biases. We have no information on the outcome of those patients with synCNS lymphoma who were considered too frail for referral to our university centers. This means that our data might be based upon a selection of those patients fit for high dosed methotrexate. Furthermore, although the rationale behind the treatment was the same, e.g. the combination of primary CNS treatment followed by systemic immunochemotherapy, there was a considerable heterogeneity in the treatment received. Some patients were treated with a sequential MBVP/R-CHOP and others with an alternating HD-MTX/R-CHOP regimen. Although we observed no significant difference between the regimens in OS, we cannot assess the separate effect of teniposide and carmustine. In addition, 7 of 21 patients were unable to complete treatment as planned (Supplementary Figure 1). Except for gender and associated testicular or breast localization we observed no significant differences in clinical characteristics or outcome. Given its rarity, sysCNS B-NHL comparative studies can only be performed in a large, international multicentre setting. Lastly, high dose cytarabine and thiothepa were not part of the treatment regimens, although in PCNSL the addition improved outcome in a randomized phase II study [7]. Whether the addition of cytarabine or thiothepa in sysCNS B-NHL is feasible remains to be established, but might be offset by the TRM.

Conclusions

In this second largest cohort of patients with sysCNS lymphoma at presentation described to date, the outcome of patients treated with HD-MTX and R-CHOP was excellent for those patients who obtained a complete remission. This challenges the fact that these patients are excluded from most trials, and usually are considered as having a dismal prognosis. Prospective studies are needed to define the optimal treatment for these patients.

Tables

Table 1. Clinical characteristics at diagnosis of the 21 patients with concomitant systemic and parenchymal central nervous system B-cell non-Hodgkin.

Characteristics	Sequential MBVP / R-CHOP	Alternating MTX / R-CHOP
Patients (n)	10	11
Median age	54 (41-60)	59 (19-71)
Gender (M/F)	2/8	7/4
B-cell lymphoma		
Diffuse large B-cell lymphoma (%)	5 (50)	9 (82)
Iatrogenic immunodeficiency lymphoma (%)	2 (20)	1 (9)
Transformed marginal zone lymphoma (%)	2 (20)	0 (0)
Intravascular B-cell lymphoma (%)	1 (10)	1 (9)
Extra CNS involvement		
Testicular (%)[a]	0 (0)	4/7 (57)
Breast (%)[a]	2/8 (25)	0 (0)
Bone / bone marrow (%)	6 (60)	2 (18)
Kidney / lung (%)	4 (40)	0 (0)
Cerebrospinal fluid involvement (%)	4 (40)	4 (45)
Elevated LDH (%)	6 (60)	6 (54)
WHO performance		
1-2 (%)	2 (20)	5 (45)
3-4 (%)	8 (80)	6 (55)
IPI-score		
1 (%)	1 (10)	1 (9)
2-3 (%)	7 (70)	7 (64)
4-5 (%)	2 (20)	3 (27)

[a] *percentage in patients at risk*

Table 2: Treatment intensity in the 21 patients with concomitant systemic and CNS B-NHL at diagnosis.

Characteristics	Sequential MTX / R-CHOP	Alternating MTX / R-CHOP
Patients (n)	10	11
Median MTX cycles (n)	4 (2-6)	5 (1-8)
Median cumulative MTX dosing (gr)	12 (6-18)	15 (3-24)
Median i.t. MTX cycles (n) [a]	6 (4-10)	9 (4-13)
Median CHOP cycles (n)	6 (0-6)	6 (1-8)
Rituximab (%)	10 (100)	10 (90)
Additional chemotherapy	Teniposide Carmustine	n.a.
Radiotherapy in partial remission (%)	3 (30)	4 (36)

[a] *in patients receiving i.t. therapy*

Figures

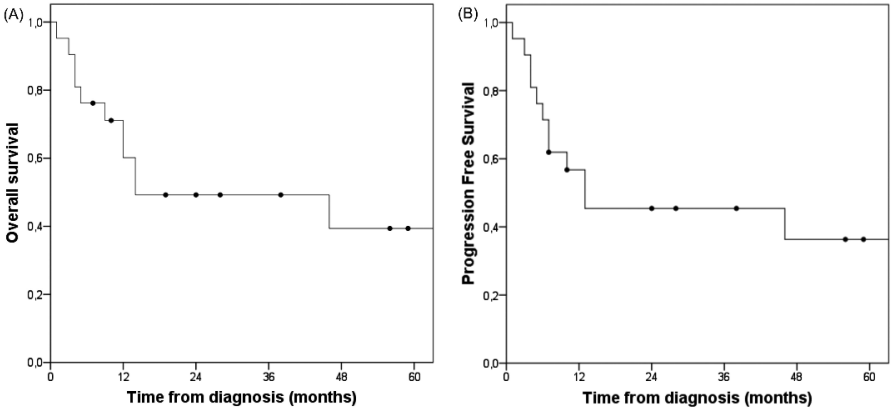


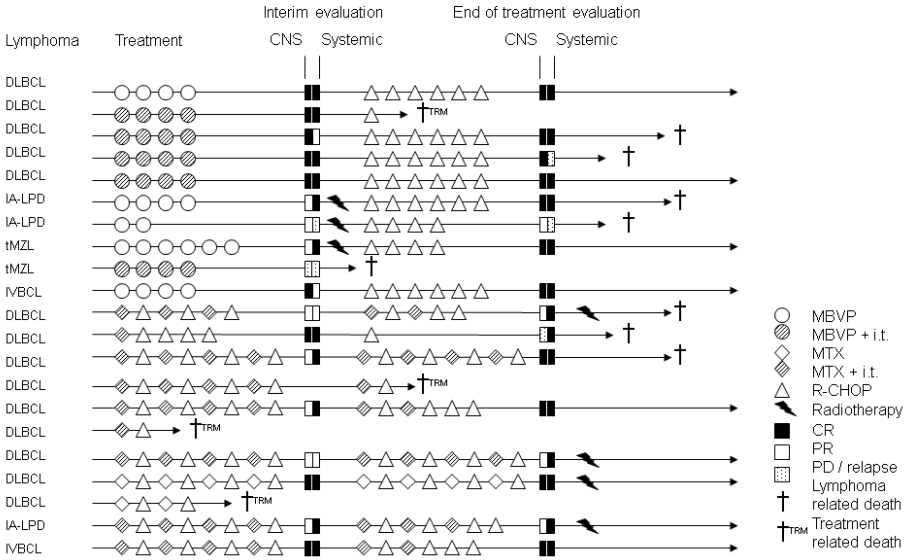
Figure 1. (A) Overall survival and (B) progression free survival of 21 patients with concomitant systemic and parenchymal CNS B-cell non Hodgkin lymphoma at diagnosis.

Supplementary

Supplementary table 1: Outcome of chemotherapeutic regimens in secondary relapsed CNS lymphoma and sysCNS lymphoma

Ref	CNS relapse	SysCNS	Patient (n)	Regimen	CR (%)	ORR (%)	OS (%)	PFS / TTF
Doorduijn (2012)	Yes	No	39/0	HD-MTX cytarabine cisplatinum ASCT	28	53	1-yr 22	1-yr 21
Korfel (2013)	Yes	No	30/0	HD-MTX thiothepa ASCT	50	57	2-yr 68*	2-yr 58*
Maciocia (2015)	Yes	Yes	13/10	HD-MTX cytarabine anthracycline	40	70	2-yr 90	2-yr 70
Ferreri (2015)	Yes	Yes	22/16	HD-MTX cytarabine ASCT	24	74	5-yr 41 5-yr 68*	5-yr 40 5-yr 62*
Damaj (2015)	No	Yes	0/60	HD-MTX cytarabine/ anthracycline ASCT	68	76	3-yr 44 3-yr 73*	3-yr 42 3-yr 73*
Current study	No	Yes	0/21	HD-MTX anthracycline	57	62	3-yr 49	3-yr 45

For treatment regimens where an autologous stem cell transplantation (ASCT) was offered to patients in PR/CR the outcome data are shown separately (*). Abbreviations: CNS relapse, relapsed central nervous system of systemic B-NHL; sysCNS, concomitant systemic and parenchymal CNS B-cell lymphoma at diagnosis; HD-MTX, high hose methotrexate; ASCT, autologous stem cell transplantation; n.a., not available



Supplementary figure 1: Detailed graphical representation of detailed treatment information, response evaluation, outcome and follow-up for 21 patients with concomitant systemic and parenchymal CNS B-NHL at diagnosis.

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Chapter 11

Combined PD-1 and JAK1/2 inhibition in refractory primary mediastinal B-cell lymphoma

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Introduction

Primary mediastinal large B-cell lymphoma (PMBL) represents 2–4% of all B-cell non-Hodgkin lymphoma [1]. It shares clinical and molecular features with classical Hodgkin lymphoma [2]. Amplification or gain of 9p24.1 is observed in more than half of PMBL cases, resulting in overexpression of the immune checkpoints programmed cell-death ligand 1 and 2 (PD-L1 and PD-L2) and the cytokine receptor signaling kinase Janus kinase 2 (JAK2) [3, 4]. Besides increased growth signaling through the JAK-STAT pathway, JAK2 overexpression enhances PD-L1 transcription and expression [3]. Although recent studies indicate a favorable outcome of PMBL patients treated with dose-adjusted etoposide, doxorubicin, cyclophosphamide, vincristine, and prednisone plus rituximab in first line [5], outcome of relapsed large B-cell lymphoma remains poor.

Material and methods

Immunohistochemistry

Tissue sections of 3 µm were cut from formalin fixed and paraffin embedded tissue samples. Immunohistochemical staining was performed according to standard procedures. Briefly, sections were dewaxed with xylene and endogenous peroxidase was blocked. Antigen retrieval was performed in 10 mM Tris (tris-hydroxymethylaminomethane) / 1 mM EDTA (ethylene diamine tetracetic acid) at pH 9.0. Staining was visualized with rabbit monoclonal antibodies PD-L1 (E1L3N) (anti-PD-L1, Cell Signaling Technology, the Netherlands) and phospho-STAT3 (Tyr705)(D3A7) (anti-pSTAT3, Cell Signaling Technology, the Netherlands) and with the mouse monoclonal antibody PD-1 (MRQ-22) (anti-PD1, Ventana, Swiss). Primary antibodies were detected by secondary and tertiary conjugate antibodies. Scoring was performed by 2 experienced hematopathologists.

Fluorescence in situ hybridization

Fluorescence in situ hybridization was performed with a CD274 / CEN9 dual color probe (ZytoLight SPEC CD274, PDCD1LG2 / CEN 9 Dual Color Probe, Zytovision, Germany) according to the manufacturer's instructions.

Ethics approval and consent to participate

The patient provided informed consent for all her sequential therapies. Written informed consent was obtained from the patient for the publication of this case report and accompanying images.

Case report

Herein, we describe a 39-year-old Caucasian female with a refractory PMBL who was treated with a novel combination of PD-1 and JAK2 inhibition. PMBL had progressed after R-CHOP. Subsequent treatments consisted of dexamethasone, cytarabine, and cisplatinum (DHAP), brentuximab vedotin, and radiotherapy. Progression was histologically confirmed (Fig. 1a). Fluorescence in situ hybridization revealed 9p24.1 amplification (Fig. 1b) and polysomy (Fig. 1c). However, PD-L1 or pSTAT3 (as a measure of increased JAK2 activity) expression could not be observed (Fig. 1d, e). PD-1 staining of T-cells (Fig. 1f) was limited. The patient had a lifethreatening vena cava superior syndrome (VCSS). In the absence of a clinical trial, with the patient's consent, she was treated with off-label ruxolitinib at a dose of 10 mg bid. Off-label pembrolizumab was initiated 2 weeks later at 2 mg per kilogram in 3-week cycles (Fig. 2a). Four weeks after start of ruxolitinib the VCSS had resolved. Staging and response evaluation were performed with combined fluorodeoxyglucose positron emission tomography and computerized tomography scans according to the Lugano classification [6]. A complete metabolic remission was documented 10 weeks after start of ruxolitinib (Fig. 2b). The patient continued treatment until an allogeneic hematopoietic cell transplantation (HCT) after non-myeloablative conditioning with fludarabine (30 mg/m2/day for 3 days) plus 2 Gy total body irradiation from an HLA identical matched unrelated donor. Besides anemia grade 1, no adverse events were observed over the course of treatment. The patient is in complete remission for 24 months, with excellent quality of life.

Discussion

Optimal treatment of relapsed PMBL has not been defined. Despite CD30 expression in 80% of PMBL cases, the anti-CD30 antibody drug conjugate brentuximab vedotin showed an ORR of only 13%.[7] In a small pilot study, the JAK 1/2 inhibitor ruxolitinib showed limited activity as a single agent.[8] With an overall response rate of 41% the efficacy of the anti-PD-1 antibody pembrolizumab in relapsed PMBL is encouraging.[9] However, complete remissions are observed infrequently and first responses are observed months after treatment initiation.[9] In contrast, the combined treatment with ruxolitinib and pembrolizumab in our patient resulted in a prompt clinical and metabolic remission. Accordingly, the addition of ruxolitinib to PD-1 blockade has been proposed to inhibit JAK-STAT proliferation and decrease PD-L1 expression on tumour cells.[3] Given the favourable toxicity profile, ruxolitinib can be applied in heavily pre-treated patients and is attractive for combination therapy.[10] Future studies are warranted to investigate the efficacy of combined PD-1 and JAK1/2 inhibition in relapsed PMBL.

Figures

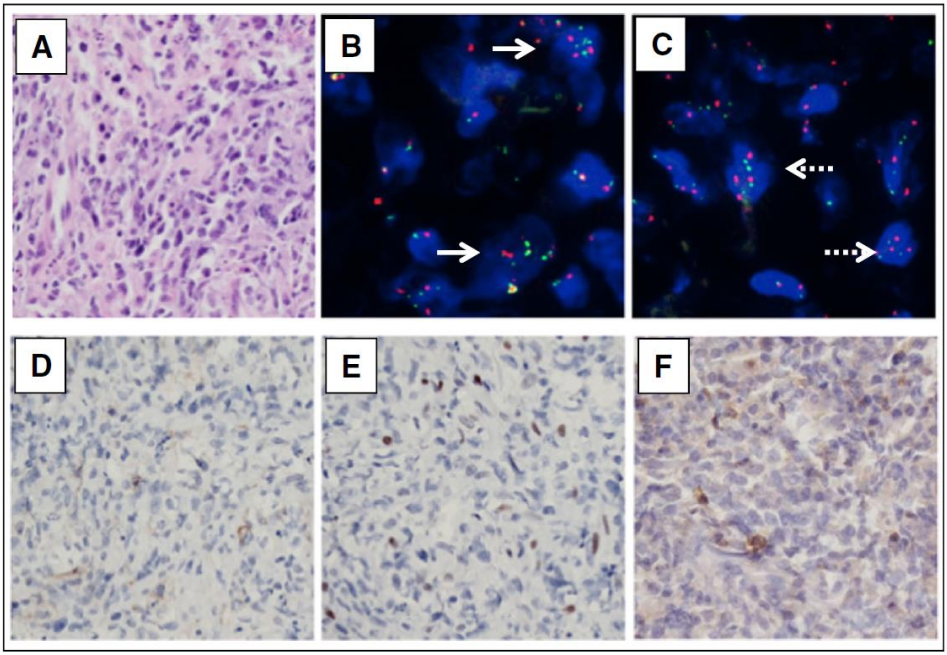

Figure 1. Immunohistochemical staining and fluorescence in situ hybridization of formalin-fixed paraffin-embedded refractory primary mediastinal B-cell lymphoma tissue for PDL-1, pSTAT3, and PD-1. **(**A) Hematoxylin and eosin staining of the paraffin-embedded mediastinal needle biopsy at progression (40×). **(**B,C) Fluorescence in situ hybridization with a CD274 (green)/CEN9 (red) dual color probe showed amplification (solid arrows) and polysomy (dashed arrow) both in 10% of tumor cells. **(**D,E) Immunostaining with anti-PD-L1 and anti-pSTAT3 of paraffin-embedded tumor biopsy revealed no staining of tumor cells (40×). (F) Immunostaining with anti-PD-1 showed modest PD-1 expression on infiltrating T-cells (40×)

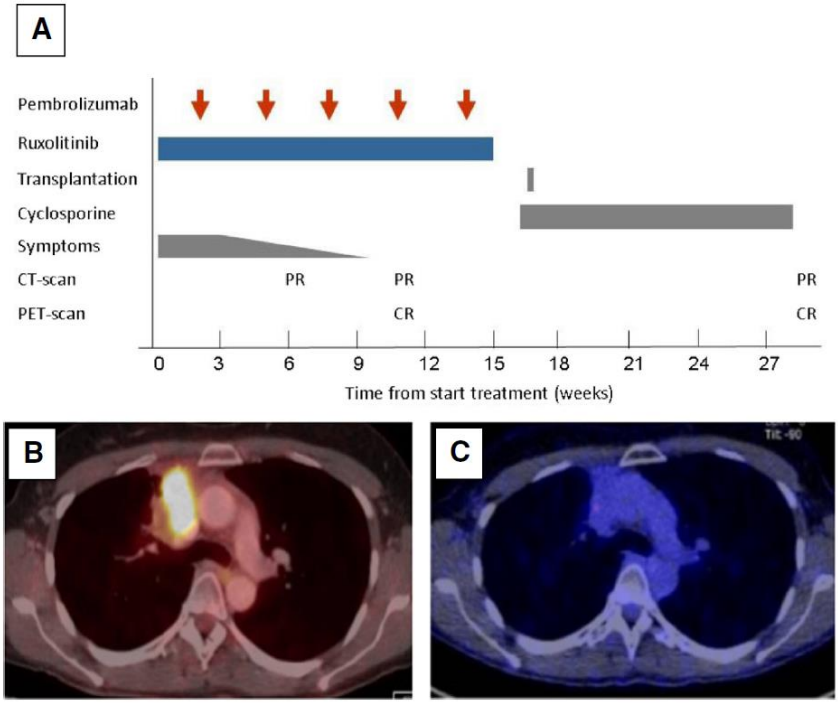

Figure 2. Treatment and response assessment of patient with a refractory primary mediastinal B-cell lymphoma treated with PD-1 and JAK1/2 inhibition. (A) Treatment schedule and response assessments show a rapid clinical and complete metabolic response. The duration of response was sufficient to perform an allogeneic hematopoietic cell transplantation (HCT), with an ongoing remission 20 months after HCT. **(**B) Combined fluorodeoxyglucose (^{18}FDG) positron emission (PET) computed tomography (CT) scans before treatment showed a metabolic active tumor in the anterior superior mediastinum with encasement of the vena cava superior and brachiocephalic vein. **(**C) ^{18}FDG PET-CT scan 10 weeks after initiation of the combined pembrolizumab and ruxolitinib treatment showed a metabolic complete remission (Deauville 3) with a residual mass. PR, partial remission; CR, complete remission

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Chapter 12

Summary, general discussion and perspectives

Summary and general discussion

Aggressive B-cell lymphomas are a heterogeneous group of mature B-cell neoplasms. Morphologically diffuse large B-cell lymphoma (DLBCL), high grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangement (HGBCL-DH) and primary mediastinal B-cell lymphoma (PMBCL) share similarities, but phenotypically and genetically there are big differences. Part of the aggressive B-cell lymphomas arise as transformed lymphoma from indolent lymphoma, with follicular lymphoma being the most prominent one. The aim of this thesis was to investigate what drives the aggressive lymphomas, to study the characteristics of relapses and to explore novel diagnostic and therapeutic options.

Pathogenic drivers

We performed proteome profiling analyses of DLBCL cell lines and compared these with immortalized lymphoblastoid cell lines (LCL). The results were confirmed in primary NHL tissues (**Chapter 2**). DLBCL cell lines had 14 recurrently downregulated proteins, 8 upregulated proteins and 10 with variable expression. Annotations of the proteins covered six hallmarks of cancer: motility (n = 6), metabolism (n = 6), chromatin modification and transcription (n = 5), anti-oxidant (n = 4), immune response (n = 4), and signal transduction and membrane transport (n = 3).[1] The anti-oxidative protein PRDX was more increased in cases with aggressive transformed follicular lymphoma (tFL) and DLBCL as compared to the indolent FL cases. This could have helped to overcome the effect of reactive oxygen species that stress tumor cells. Somewhat surprisingly we found PPIA, the target for the immunosuppressive drug cyclosporine, to be elevated. We showed that growth of these cell lines could be blocked by cyclosporine A. Cyclosporine has never been used as therapy in lymphomas, outside angioimmunoblastic T cell lymphoma, in which it was rather effective.[2] The clinical application of cyclosporine, however, might be outbalanced by the immune suppressive effects of cyclosporine and its association with post-transplant lymphoproliferative disorders (PTLD).[3]

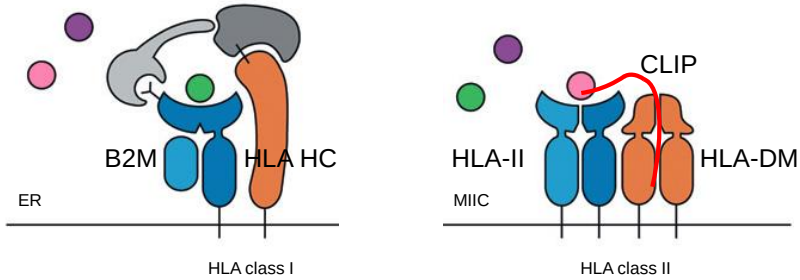
We showed that nearly 40% of tFL cases had a *MYC* rearrangement (**Chapter 3**). Our data support the findings of MYC being a strong driver of transformation of FL.[4,5] Although the number of *MYC* rearranged cases was limited, our data seem to indicate that *Ig-MYC+* rearrangements result in stronger upregulation of MYC than non-Ig rearrangements.[6] In 2018 Chong *et al*. showed that the breakpoint of Ig-MYC+ cases clustered in intron 1, whereas the non-*Ig MYC+* cases were characterized by breakpoints downstream of the *MYC* gene with a variety of translocation partners, including *BCL6*, *ZCCH7* and *RFTN*.[7] By immunohistochemistry we found higher MYC expression in FL grade 3B than in FL grade 3A and FL1/2, and even higher expression in tFL. Immunohistochemistry showed MYC staining in >90% of tumor cells in *Ig-*

MYC+ cases and a more variable staining in non-*Ig MYC*+ cases, with only 1 *MYC* rearranged case with MYC IHC <40%. Hence, a MYC expression of < 40% has a good negative predictive value for excluding *MYC* rearrangements. However, nearly half of the tFL cases without a *MYC* rearrangement also had high expression of MYC, suggesting alternative mechanisms of MYC activation, which limits the positive predictive value of *MYC-IHC* for the selection of *MYC* rearranged cases. Recent studies seem to indicate that irrespective of the cause of MYC overexpression, dual expression of MYC and BCL2 by itself might be a negative prognostic factor for survival.[8,9] In addition, Ennishi *et al*. published in 2018 a study on a double hit gene expression signature, which has a strong correlation with the dual IHC expression. These data seem to indicate that a subgroup with an unfavorable outcome can be identified within the GCB-type DLBCL.[10]

Immune evasion

One of the hallmarks of both DLBCL and PMBCL is immune evasion. In this thesis we showed that there is a frequent defect in the cell surface expression or functioning of HLA class I and class II of aggressive B-cell lymphomas. In addition, we observed a novel functional defect of antigen presentation that is characterized by retention of the invariant chain in HLA class II, due to loss of HLA-DM (**Chapter 4**) (Figure 1). Interestingly we also found CALR, the chaperone molecule for HLA class I, to be differentially expressed in DLBCL as compared to normal LCL (**Chapter 2**).

What underlies the HLA losses in DLBCL is only partially understood. In PMBCL rearrangements of CIITA causing downregulation of the HLA molecules are frequent [11], whereas in primary CNS lymphoma (PCNSL) and testicular DLBCL loss of HLA molecules is mainly caused by the loss of the HLA locus.[12] Loss of β2M partially explains the loss of HLA class I (**Chapter 2**). Recently, loss of HLA class I, but not HLA class II, was related to MYC gene expression.[10] HLA class II loss might be caused by epigenetic mechanisms related to differentiation of post-germinal B cells to plasma cells (**Chapter 5**). Looking at DLBCL cases that relapsed after R-CHOP, we observed an enrichment of mutations of genes involved in antigen presentation, including the HLA molecules and immunoglobulin light chains, which further emphasizes the importance of immune escape in pathogenesis and therapy resistance (**Chapter 7**).



	β-2 microglobulin	HLA-I heavy-chain	α-chain β-chain	CLIP
DLBCL	Mutations	Loss	Epigenetic	Loss of HLA-DM
HL		Mutations in CIITA		
PMBCL				
PCNSL	Loss of HLA locus at chromosome 6p21			

Figure 1: Schematic representation of potential defects in antigen presentation in DLBCL, HL, PMBCL and PCNSL. Defects in HLA class I, HLA class II and HLA-DM with the underlying mechansims are shown. Abbreviations: B2M, beta 2 microglobuline; HLA, human leukocyte antigen; HC, heavy chain; ER, endoplasmic reticulum; MIIC, MHC class II loading compartment; CIITA, class II MHC complex transactivator; CLIP, Class II-associated invariant chain peptide.

Previous *in vitro* studies showed that many of these losses lead to diminished tumor infiltrating lymphocytes and adverse prognosis.[13,14] In 2018 Roemer *et al*. published their study on the prognostic impact of HLA and PD-L1 in Hodgkin lymphoma patients treated with the checkpoint inhibitor nivolumab.[15] Complete responses were correlated with HLA class II expression. Loss of HLA class II, but not of HLA class I, was an adverse prognostic factor for progression-free survival in patients treated > 12 months after autologous stemcell transplantation. Interestingly, in other malignancies, restoration of HLA class I and class II elicited anti-tumor responses. In metastatic Merkel cell carcinoma, treatment with interferon gamma or the hypomethylating agent azacitidine could restore the loss of HLA class I.[16] In samples from relapsed AML patients, treatment with interferon gamma rapidly induced re-expression of HLA class II.[17] Whether HLA restoration is possible and if this has functional consequences in B-cell lymphomas has to be established. Moreover, this might be complicated as HLA does not stand alone in a very complicated immunological synapse. Among perturbations of this immunological synapse frequent loss or alteration of cell adhesion molecules (CD11a, CD18, CD54, and CD58), co-stimulatory molecules (CD80 and CD86) or checkpoint inhibitors (PDL-1, PDL-2, CTLA-4, and soluble HLA-G) in DLBCL should also be mentioned (Figure 2).[18-27]

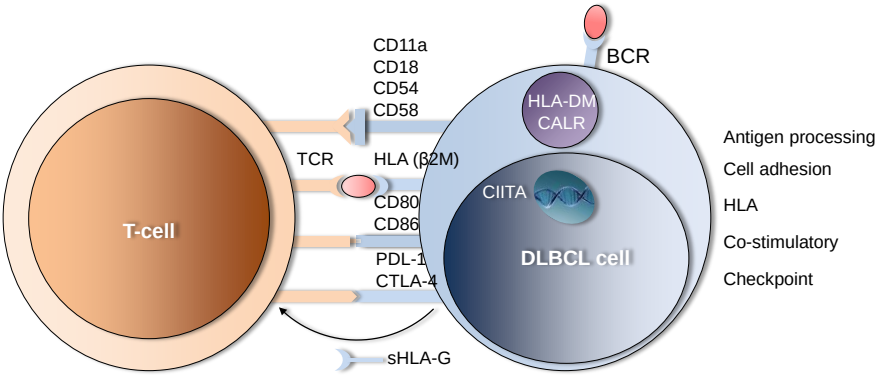


Figure 2: Molecules in the immunological synapse known to be altered in diffuse large B-cell lymphoma. Abbreviations: BCR, B-cell receptor; TCR, T cell receptor; HLA, human leukocyte antigen; PDL-1, program death ligand 1; CTLA-4, cytotoxic T-lymphocyte–associated antigen 4; sHLA-G, soluble HLA-G; CIITA, class II, major histocompatibility complex, transactivator.

Characteristics of relapse

In contrast to data on primary tumor samples, the knowledge on the characterics and biology of relapsed tumors is limited. In this thesis we explored relapses from patients who were considered to have a favorable prognosis at presentation, and relapses from advanced stage disease.

Patients with stage I (E) DLBCL generally have an excellent outcome. Remarkably, data on the treatment and subsequent outcome of relapsing patients with limited stage DLBCL are scarce. Recently, the German study group presented their data on reducing the number of cycles of R-CHOP to 4 in patients with limited stage DLBCL and an IPI-score of 0.[28] We and others have shown that de-escalation of therapy might not be optimal for all patients with stage I DLBCL (**Chapter 6**).[29] We observed that outcome of patients with relapse after stage I disease is generally poor, in part because the severity of the relapse is underestimated, and in part due to unfavorable clinical and biological factors that occur at relapse. Amongst these, central nervous system (CNS) relapse and the occurrence of *MYC* translocations seem important. The question is whether relapses could have been prevented by offering additional therapy but based on current evidence this is not likely. Firstly, Miller *et al*. already showed that eight CHOP cycles were as good as three cycles plus involved field radiotherapy, thereby not preventing less peripheral relapses.[30] Secondly, additional cycles of R-CHOP are unlikely to benefit patients with HGBCL-DH.[31] Finally, rituximab has limited activity in CNS lymphomas.[32]
While currently over 2000 primary DLBCL cases have been sequenced, data on the mutational

evolution in relapsed DLBCL is limited to small series. [33-36] To add to this data, we performed an explorative sequencing study in primary-relapse pairs (**Chapter 7**). We encountered the problem that obtaining good material from paired tumor samples is challenging, due to the omission of re-biopsies in patients with progressive disease, the tendency to perform fine needle biopsies, and degradation of DNA in formalin fixed paraffin embedded material, thereby making it unsuitable for the use of whole exome sequencing. In four out of six paired tumor samples we observed limited clonal changes, whereas in the other two patients there was a much larger shift in mutational load. This is in line with observations of early and late divergent models, which have different mutational dynamics.[33,34,36] In the early divergent model the relapse tumor evolves from a more distant progenitor and hence has more clonal drift. The late divergent model follows a more linear course. Which model occurs most frequent and how this will impact the development of targeted therapy – the target being the product of a new clonal change - are unexplored territories in DLBCL. As a proof of concept, Scherer *et al*. have shown that circulating tumor DNA in DLBCL can be used for tracking genes that potentially get mutated over time.[36] Tracking of specific genes, however, implies that the mechanism of tumor evasion can be foreseen. While this might be the case for some drugs, the mechanisms of resistance are often not known. The most frequent base pair substitution (C:G > T:A) in mutations exclusively present in the relapse samples can arise as a consequence of canonical AID activity, but it is also the most frequently observed base pair change caused by cyclophosphamide. We identified genes possibly related to therapy resistance, including tyrosine kinases, (transmembrane) glycoproteins, and genes involved in the JAK-STAT pathway. Among the potentially resistance-related genes were *PIM1* and *SOCS1*, which were present in 5 of 6 patients. These genes have been reported to convey a risk for treatment failure and might be targets for therapy.[37,38] So far, the results of the pan-PIM inhibitors AZD1208 and LGH447 as monotherapy showed only very limited responses in AML, multiple myeloma and solid malignancies.[39,40] However, preclinical data suggests that PIM inhibitors sensitize cancers to chemotherapy and tyrosine kinases and thus has the potential for combination therapy.[41,42]

Diagnostics

Tumor cells need to adapt to sustain their metabolic demands. While ^{18}F-FDG is now a well-established tool in the staging and evaluation of lymphoma patients [43], we were interested whether HGBCL-DH would have a stronger metabolic PET signature, with MYC driving metabolism. However, we did not observe a difference for semi-quantitative PET characteristics in HGBCL-DH and DLBCL (**Chapter 8**). We did, however, observe an interesting phenomenon. It appeared that necrosis was present in 25% of cases and that this was an independent negative prognostic factor irrespective of rearranged MYC status. As always, one has to be careful in interpreting scan results. ^{18}F-FDG PET-scans can be subject to false positive results (**Chapter 9**).

Treatment

Treatment has largely remained unchanged during the last 20 years. In light of the studies in the '90s current attempts with more intensive chemotherapy seem unlikely to be beneficial. One important thing that should not be overlooked, is the risk of CNS relapse. Methotrexate (MTX) might be beneficial for patients at increased risk of CNS relapse. Although data from the phase 3 trial of systemic versus intrathecal MTX have to be awaited (NCT02777736), we have shown in **Chapter 10** that even in patients with concomitant systemic and CNS localization at diagnosis, the combination of R-CHOP and high dose MTX is an effective regimen. In contrast, patients with a CNS relapse after R-CHOP generally have a very poor outcome, even after initial stage I disease (**Chapter 6**).[44,45] Although the use of very high dose chemotherapy followed by autologous stem cell transplantation (ASCT) is advocated in this setting by Ferreri *et al*, the data supporting this strategy are limited.[46] We and recently others have shown a 2-year overall survival of 40-70% with or without ASCT.[47,48] Even without the use of ASCT, toxicity is high compared to that seen in DLBCL without CNS involvement or in primary CNS lymphoma with a treatment-related mortality of 10-15%.[47,48]

In PMBCL the use of the more intensive regimen - DA-EPOCH-R - was first published in 2013.[49] The very favorable outcome with long-term survival in excess of 90% of this rather toxic DA-EPOCH-R has, however, not been confirmed thus far. The current advantage of DA-EPOCH-R over R-CHOP is the omission of radiotherapy in patients achieving a complete remission. Data of the phase 3 comparative International extranodal lymphoma study group (IELSG) study assessing the role of mediastinal radiotherapy after rituximab- containing chemotherapy regimens have to be awaited.[50] Irrespective of the results, novel less toxic therapies are needed. Brentuximab vedotin, targeting CD30, might be such a candidate. Despite the high efficacy in Hodgkin lymphoma and CD30 positive T-cell lymphoma [51-53], as well as encouraging results in relapsed B-NHL with variable CD30 expression [54], brentuximab vedotin was ineffective in PMBCL, which has CD30 expression in 80% of cases.[55] Amplification or gain of 9p24.1 is observed in more than half of PMBL cases, resulting in overexpression of the immune checkpoints programmed cell-death ligand 1 and 2 (PD-L1 and PD-L2), and the cytokine receptor signaling kinase Janus kinase 2 (JAK2).[56] Monotherapy with checkpoint inhibitors showed high overall response rates in both HL and PMBCL, but complete remission rate was low.[57,58] Based upon *in vitro* synergistic activities, we describe the successful treatment of a patient with a refractory PMBCL with the novel combination of the JAK2 inhibitor ruxolitinib, which on its own has limited activity, and the anti-PD1 checkpoint inhibitor pembrolizumab (**Chapter 11**). The potential therapeutic effect of this combined treatment could be counterbalanced by the negative effect of JAK1/2 inhibition on T-cell activity.[59] However, this might be limited as patients receiving ruxolitinib after allogeneic hematopoietic stem cell transplantation maintain effective graft versus leukemia

responses.[60] Given the favorable toxicity profile, ruxolitinib can be applied in heavily pre-treated patients, thus making it an attractive candidate for combination therapy for patients with lymphomas with a deregulated JAK-STAT pathway.[60]

Perspectives – from bench to bedside

The contents of this thesis are linked to several ongoing studies. These studies are driven by 3 key questions: 1) how can we define biological subgroups, 2) how and when can specific biological subgroups be targeted, and 3) how can we improve treatment strategies.

Biological subgroups

The optimal biological classification for DLBCL has not been established, but presence of a *MYC* rearrangement with a *BCL2* and/or *BCL6* rearrangement is well accepted to be a subgroup with an unfavorable outcome and is now part of routine diagnostics (Figure 3). Although the cell of origin (COO) within DLBCL is well established, the clinical trials with R-CHOP plus a targeted drug within a specific COO group have failed. This does not necessarily mean that the COO groups are not important. It could be that either the drugs used in combination with R-CHOP are insufficient or that the COO group should be refined. Based on consensus clustering, immune- and stromal background signatures, and *MYC* rearrangement status, it is likely that there are additional subgroups within the ABC and GCB-type DLBCL. [10,61-64] How to implement the mutational data in clinical practice is still unclear. Although over 2000 DLBCL cases have been analyzed and some genes are more frequently mutated in the ABC or in the GCB subtype, the optimal clustering into biological subgroups is still a matter of debate.[37,38,65,66] Conformational studies, like the biological analysis of the HOVON 84 (a phase 3 study on the intensification of rituximab in DLBCL), will be necessary to solve this debate. [67] One important hurdle to complete the genomic classification, would be to capture the spatial evolution of a heterogeneous disease like DLBCL, as mutational analysis is usually performed on single tumor biopsies and hence subject to sampling error. This problem may be solved by analyzing circulating tumor DNA, that is released by apoptotic tumor cells derived from lymphoma cells throughout the body, thereby better reflecting any potential tumor heterogeneity within a patient.[36,68-70]

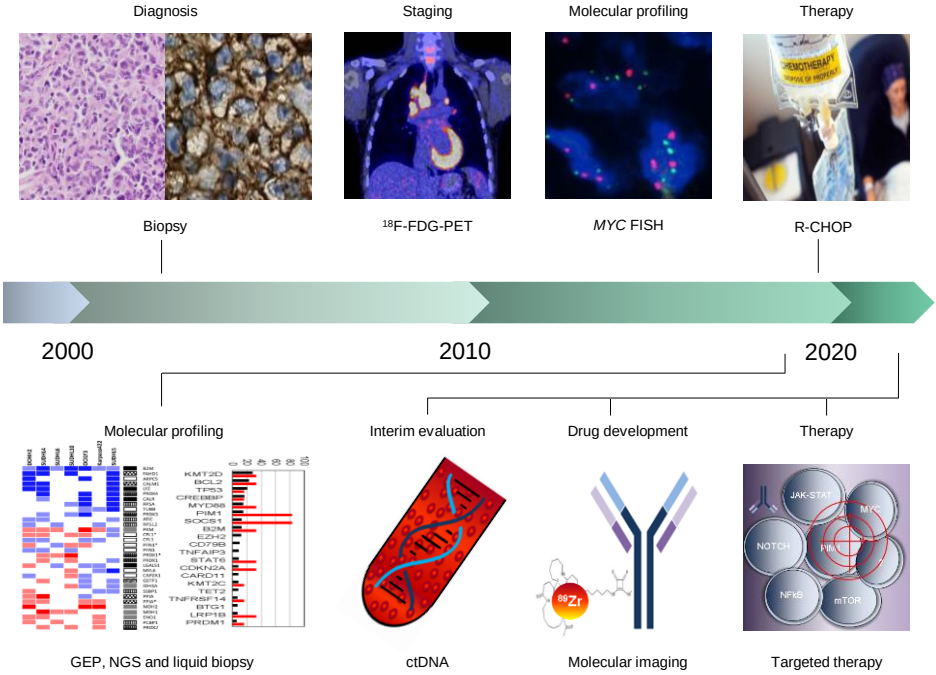


Figure 3. Schematic overview of the current diagnostic procedures and therapy in aggressive B-cell lymphoma (upper line). Diagnostic procedures that will likely impact the diagnostic process and clinical trials in the near future are molecular profiles, liquid biopsy and molecular imaging (lower line).

Targeting

When consensus is reached on the molecular subgroups, we will need to identify patients who will benefit from escalating therapy. This avoids overtreatment of low risk patients who are likely to be cured with R-CHOP, not only limiting additional toxicity as was observed with the combination of ibrutinib and R-CHOP [71], but also keeping the costs of healthcare in the Netherlands, with an annual budget of 100 billion euro, within bounds.[72]

In Hodgkin lymphoma, the use of interim ^{18}F-FDG PET scans has been very useful in the setting of escalation and de-escalation trials.[73,74] Although the negative prognostic value of interim-PET in DLBCL is generally good, the positive predictive value is rather low, thereby limiting the use of interim PET in treatment decisions.[75]

Recent studies have shown the potential of circulating tumor DNA (ctDNA) as a tool of interim and end-of-treatment analysis. Not achieving an early molecular response, defined as a 2 to 3 log fold reduction of ctDNA load within 2 courses of R-CHOP, is strongly related to progressive disease or relapse.[36,68-70] ctDNA based analysis of minimal residual disease (MRD) can be used for de-escalation studies, escalation studies early in the course of treatment or consolidation strategies after induction therapy.[76] Thus far, there is no consensus on which technique and gene panels have to be used. For each technique, ctDNA analysis will put high demands on the infrastructure and will be technologically demanding. If successfully implemented, the combined interim PET and interim ctDNA analysis could be a potential tool to guide therapy (Figure 3).[69] We have set up a national multicenter study to examine the combined use of ^{18}F-FDG-PET/CT and ctDNA-based minimal residual disease monitoring for the response assessment in post-transplant lymphoproliferative disorder (NTR 7402 / NL 7203) (Figure 4). With the successful implementation of *MYC* FISH screening in the Netherlands [77], we now need to organize the infrastructure for ctDNA analysis.

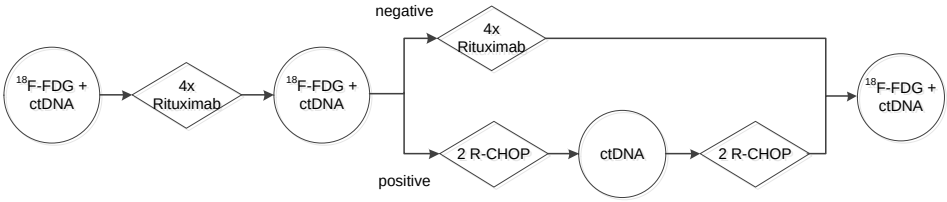


Figure 4. Scheme of ctDNA based MRD monitoring in PTLD (NTR7402). ctDNA based analysis will be performed in parallel with standard of care ^{18}F-FDG-PET scans and treatment.

Evidently, identifying patients at high risk of treatment failure early during therapy is still no guarantee to success, as was shown in the PETAL study.[78] What we need is the development of effective drugs for salvage. The most obvious candidates of new salvage drugs are small molecules targeting the NF-kB, PI3K-m-TOR, JAK-STAT and NOTCH signaling pathways, B-cell lineage markers, epigenetic modifiers or immune therapy.[79,80] Targeting the signaling pathways in first line with single agents (ibrutinib, bortezomib, everolimus) has not been successful. [71,81,82]. In the HOVON 130 addition of lenalidomide to R-CHOP showed promising results for HGBCL-DH.[69]. Given the acquisition of mutations or activation of alternative pathways, it is unlikely that targeting just one pathway will be sufficient given the complexity of DLBCL. Even with response rates of 40% of ibrutinib in relapsed ABC-type DLBCL, the short duration of response and the absence of a plateau made it improbable that it would increase the plateau of the survival curves in first line. Fortunately, sometimes new drugs do have a good outcome in the relapse setting and may become important candidates for implementation in first-line therapy. A good

example is the successful addition of brentuximab vedotin to CHP in relapsed anaplastic large T-cell lymphoma, where brentuximab vedotin resulted in a clear plateau in survival after 12 months.[53,83] In the HOVON 136 brentuximab vedotin is investigated in combination with DHAP for patients with relapsed CD30+ DLBCL, a regimen that was highly efficacious in patients with chemo-resistant Hodgkin lymphoma.[84] Another promising antibody drug conjugate (ADC) is polatuzumab vedotin targeting CD79. Patients with a relapsed DLBCL had a significantly longer progression free survival when treated with polatuzumab plus bendamustine compared to bendamustine alone (hazard ratio 0.34).[85] At 12-months the overall survival was 50%, which rivals the results of CAR-T therapy. An advantage of polatuzumab vedotin is that CD79 is universally expressed on all malignant and normal B-cells, but this does not necessarily mean that all DLBCL subgroups will benefit. In AML the efficacy of the ADC gemtuzumab ozogamicin was at least partially diminished due to upregulation of drug efflux pumps.[86] Furthermore, loss or downregulation of cell surface antigens might induce resistance, as has been observed in drugs targeting CD19, CD22 and CD52.[87-89]

While most studies aim at improving efficacy of R-CHOP, nearly 50% of patients who relapse, do so after achieving a complete remission. In patients with an IPI-score of ≥ 3 the 2-year change of relapse is 21%.[90] Patient relapsing within a year after R-CHOP treatment have a similar poor prognosis as patients with progressive disease. In relapsed DLBCL, checkpoint inhibitors show modest efficacy.[91,92] However, treatment at overt relapse might not be the right moment, as these tumors are highly proliferative. Since steroids dampen the effect of checkpoint inhibitors [93], combination of checkpoint inhibitors with R-CHOP (NCT02596971) seems less attractive.[94] Therefore, we think that the best moment to treat these patients is in a state of minimal residual disease. To this end we designed and started the HOVON 151 trial (NCT02596971), in which patients with an IPI score of ≥ 3 are treated with the anti-PDL1 antibody atezolizumab for a 12-month consolidation period (Figure 5). These patients will be monitored for dynamics of MRD at various stages of treatment and observation [68], tumor characteristics, alterations of the immune repertoire [95], microbiome [96,97] and in case of relapse clonal evolution.[36,70] In a similar way, patients with a HGBCL-DH will receive the anti-PD1 antibody nivolumab after induction therapy with DA-EPOCH-R in the HOVON 152 trial.

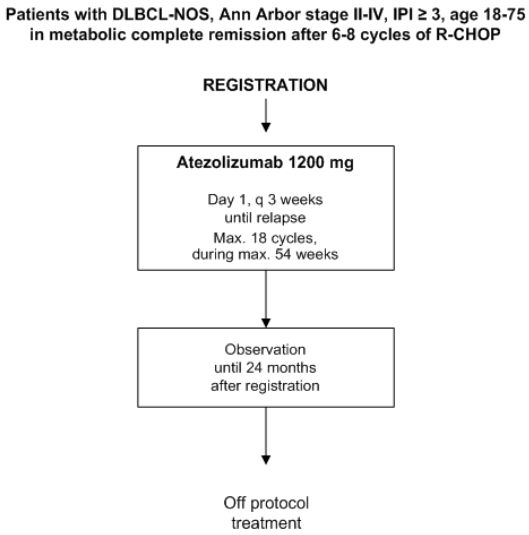


Figure 5: Scheme of the HOVON 151 study (NCT02596971). Patients with high risk DLBCL (IPI-score > 2) have >20% change of relapse even after achieving a metabolic complete remission. Eligeble patients (n = 114) will be treated with atezolizumab for the duration of 1 year.

Improving treatment strategies

As discussed previously, several drugs have failed to improve first line treatment. Apart from the financial consequences, these studies potentially put patients at risk of additional toxicities. Therefore, drug development needs to be improved. One can do so by executing innovative biological driven studies, e.g. with a Bayesian design in which patient allocation across various (biological) study arms is modeled based on results acquired during the trial.[98] However, these are complex and challenging studies that require a good infrastructure, multiple pharmaceutical partners and robust biological and statistical support.

Another important issue is that a translational program (from bench to bedside) needs to be an integrated part of clinical studies. As previously mentioned, this not only requires additional funding, but also proper specimen collection and biobanking. To that end the collection of ctDNA can improve the way we study clonal evolution and potential mechanisms of resistance. Obviously, this does not replace the need for collection of proper tumor tissue.

Finally, we consider molecular imaging using immune-PET could be a valuable tool in drug development. Molecular imaging is the noninvasive assessment of radioisotope labeled monoclonal antibodies and ADCs. Unlike conventional pharmaco-kinetics, immune-PET provides detailed *in vivo* information on biodistribution, tumor uptake and optimal dosing of a drug. A wide

variety of antibodies and ADCs have been studied in different cancers including breast, colon, prostate, ovarian and neuroendocrine tumors, and also in lymphoma.[99] Clinical applications of immune-PET include improved staging prior to therapy [100,101], monitoring of tumor uptake over time in individual patients [102], assessment of drugs interactions [103], dose finding [104,105], and prediction of response to therapy.[106] Zirconium-89 (^{89}Zr) is the radioisotope that lends itself best for visualizing antibodies and has been used in clinical trials with over 15 different monoclonal antibodies.[99]

As part of the HOVON 136 trial we will perform a molecular imaging study using ^{89}Zr brentuximab. While CD30 expression by IHC is generally required for participation in trials, there is no clear correlation between expression of CD30 and efficacy of the ADC.[52,107] Possible explanations might be tumor heterogeneity, uptake and subsequent release of the ant-tubulin MMAE in the tumor micro-environment or a threshold below the sensitivity of immunohistochemistry for detecting CD30.[54] Therefore, in the HOVON 136 ^{89}Zr brentuximab biodistribution will be related to CD30 protein and gene expression, to identify which patients benefit from brentuximab vedotin. Application of molecular imaging in the negative trial of brentuximab vedotin in PMBCL could potentially have helped to understand the mechanism of failure of therapy.[55]

As part of the HOVON 151 trial, we will perform a molecular imaging study using sequential ^{89}Zr atezolizumab PET scans (NCT03850028)(Figure 6). While it has been observed that the percentage of PDL1 positive DLBCL seems relatively low compared to other cancers (ranging from 13 to 31%), PDL1 amplifications can be observed in 39% and 25% of non-GCB and GCB DLBCL, using ctDNA analysis.[108-110] Despite higher overall response rates in PDL1 positive malignancies compared to PDL1 negative tumors, responses are seen in PDL1 negative patients.[111,112] PDL1 status from resected specimens shows poor correlation to the PDL1 status from matched biopsies.[113] Variable expression of PDL1 can be observed in multiple tumor biopsies collected over time and/or from different anatomical sites in individual patients and can be influenced by treatment.[114,115] Therefore, PDL1 expression assessed by one single biopsy probably is not representative, hence there is a need for a better predictive marker. To this end we will perform sequential ^{89}Zr-atezolizumab PET scans, that will provide information on the dynamics of atezolizumab biodistribution over time. In solid malignancies ^{89}Zr-atezolizumab PET scans correlated better with tumor responses than PDL-1 immunohistochemistry or gene expression profiles.[116]

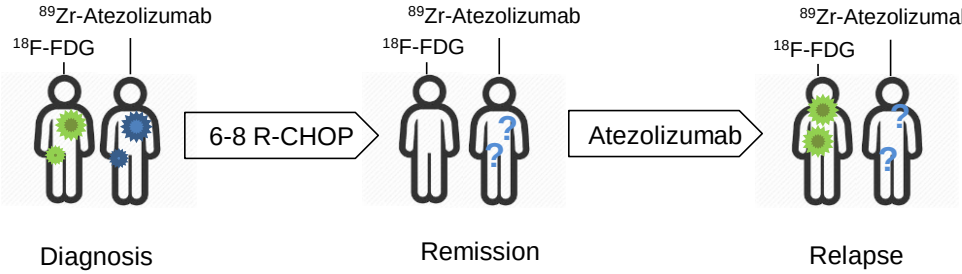


Figure 6: Flowchart of the HOVON 151 imaging side study with ^{89}Zr-atezolizumab (NCT03850028). Biodistribution will be assessed before and after R-CHOP and in the event of suspected relapse. Data will be related to PDL1 expression (IHC and GEP), minimal residual disease and possible toxicity. ^{89}Zr-PET scans at relapse might give valuable information on the mechanism of resistance.

In summary, R-CHOP has been the standard of care for patients with aggressive B-cell lymphoma in the last 2 decades. During the last several years there is a tendency to apply more intensive chemotherapy regimens for PMBCL and HGBCL-DH, but the effectiveness of this approach is not yet supported by randomized trials. The mere fact that HGBCL-DH has a poor outcome, doesn't automatically mean that more chemotherapy is the solution. Although much has been learned about the biology and genomic alterations in aggressive B-cell lymphoma, the clinical impact of the cell of origin, mutational classifications and affected pathways currently hold no clinical implications. Conformational studies based on tumor and liquid biopsies are needed to more robustly define molecular subgroups that have a meaningful prognostic impact. In combination with interim ^{18}F-FDG PET scans, analysis of circulating tumor DNA has the potential to guide treatment decisions. In order to move on we need to find the Achilles' heel of DLBCL either by improving induction or by eradicating minimal residual disease through consolidation strategies. Treatment strategies might be improved by smarter study design and solid translational research, including molecular imaging, which allows the study of the biodistribution of monoclonal antibodies and antibody drug conjugates *in vivo*. This might help to select patients who benefit most, could give information on toxicity, and indicate mechanism of resistance. These diagnostic and therapeutic considerations form the basis for several of the ongoing trials.

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Appendices

Nederlandse samenvatting

Publications

Acknowledgements

Nederlandse samenvatting

Agressieve B-cel lymfomen

Jaarlijks wordt er bij zo'n 4500 mensen in Nederland de diagnose lymfklierkanker (lymfoom) gesteld. Lymfklierkanker hoort daarmee tot de 10 meest voorkomende vormen van kanker. Lymfklierkanker is echter niet één ziekte, maar kent vele typen. Het Hodgkin lymfoom (HL) is een B-cel lymfoom dat al in 1833 als aparte ziekte is beschreven. Nadien zijn de andere vormen van lymfklierkanker als non-Hodgkin lymfomen (NHL) geclassificeerd. Door voortschrijdende biologische inzichten zijn er sindsdien meer typen NHL te onderscheiden en is de indeling van de lymfomen in de loop der jaren geregeld aangepast. In de meest recente World Health Organisation (WHO) classificatie uit 2017 worden er meer dan 30 verschillende vormen van B-NHL onderscheiden. De B-NHL kunnen ingedeeld worden in laaggradige (indolente) en hooggradige (agressieve) lymfomen. De laaggradige lymfomen zijn over het algemeen goed te behandelen met immuno-chemotherapie, maar hebben sterk de neiging terug te komen (recidiveren). Bij een deel van de patiënten met een laaggradig lymfoom treedt een verandering op, waardoor het een hooggradig lymfoom wordt (transformatie). In tegenstelling tot de indolente lymfomen is de opzet van de behandeling bij patiënten met een agressief lymfomen om hen te genezen. Helaas is de keerzijde dat recidieven veelal niet goed te behandelen zijn en frequent fataal aflopen. Er worden momenteel 4 agressieve B-NHL onderscheiden: het *diffuus grootcellig B-cel lymfoom* (DLBCL, 37%), *het primair mediastinaal B-cel lymfoom* (PMBCL, 3%), het *hooggradig B-cel lymfoom met een MYC breuk en een BCL2 en/of BCL6 breuk, ook wel dubbel of triple hit lymfomen genoemd* (HGBCL-DH, 2.5%) en het in Nederland zeldzame *Burkitt lymfoom* (BL, 0.8%). Binnen het DLBCL worden er nog enkele specifieke subtypen onderscheiden, zoals het primair grootcellig B-cel lymfoom van de hersenen, maar in de meerderheid van de gevallen is het (vooralsnog) niet nader te classificeren. Ondanks dat de agressieve B-NHL gemeenschappelijke overeenkomsten hebben, zijn er kenmerkende verschillen in de ontstaanswijze, de klinische presentatie, prognose en behandeling. De laatste 10 jaar zijn er nieuwe technieken gekomen voor diagnostiek, classificatie en respons beoordeling. Biologische inzichten hebben geleid tot de ontwikkeling en registratie van verschillende nieuwe medicijnen bij de laaggradige B-NHL. Echter, bij de behandeling van patiënten met een agressief B-cel lymfoom hebben deze inzichten nog niet geleid tot een verbetering.

Het doel van dit proefschrift is om te onderzoeken welke additionele factoren bijdragen aan het ontstaan van agressieve lymfomen en wat de eigenschappen zijn van DLBCL die recidiveren. In dit proefschrift wordt onderzocht of er een relatie is tussen biologische karakteristieken en positron emission tomography (PET) scans en we delen de ervaring van 2 nieuwe behandelstrategieën.

Etiologie van agressieve lymfomen

De afgelopen decennia is er veel vooruitgang geboekt in het onderzoek naar de ontstaanswijze (etiologie) van de B-cel lymfomen. Zo is duidelijk geworden dat de verschillende B-NHL terug te voeren zijn tot de cel-van-oorsprong (COO). Onder normale omstandigheden heeft een B-cel (B-lymfocyt) verschillende stadia die doorlopen wordt vanaf het herkennen van een lichaamsvreemd eiwit (antigeen) tot het produceren van afweerstoffen (antistoffen). Tijdens de selectie en uitrijping van een B-cel kan een defect ontstaan, waardoor dit proces wordt verstoord. De defecte B-cel zal geprogrammeerd dood gaan (apoptose) of door het immuunsysteem worden herkend en uitgeschakeld. Echter, indien de defecte B-cel additionele veranderingen ondergaat in het DNA (mutaties) of er veranderingen in de eiwitten (epigenetisch) ontstaan, kan deze ontaarden in een tumorcel. B-cellen hebben in tegenstelling tot andere tumoren een unieke eigenschap. Voor de normale functie van de B-cellen is het belangrijk om zoveel mogelijk verschillende antistoffen te kunnen maken. Hiertoe moet het stuk DNA wat codeert voor de antistoffen, het zware immunoglobuline locus (IgH), door de B-cel veranderd kunnen worden. B-cellen brengen hiervoor speciale eiwitten (RAG en AID) hoog tot expressie. Helaas veroorzaken deze eiwitten soms ook breuken en mutaties op plaatsen in het DNA buiten het IgH locus. Daarmee kan in beginsel ook een B-NHL ontstaan. B-NHL's zijn dus het gevolg van fouten in de ontwikkeling tijdens verschillende ontwikkelingsstadia van de normale B-cel.

Op basis van deze inzichten is duidelijk geworden welke eiwitten en signaaltransductie systemen (pathways) belangrijk zijn. Zo wordt er binnen het DLBCL op basis van gen-expressie profielen (GEP) onderscheid gemaakt tussen het germinal center type (GCB) en het geactiveerde B-cel type (ABC). Daarnaast is duidelijk geworden welke zeer uiteenlopende chromosoom veranderingen (rearrangements) en veranderingen in het DNA (mutaties) voorkomen. Een van de meest in het oog springende chromosoomverandering betreft het *MYC* gen, gelegen op chromosoom 8. Een verandering hierin kan worden vastgesteld bij 10-15% van de DLBCL patiënten. Dit is belangrijk, omdat patiënten met een HGBCL-DH helaas een slechte prognose hebben.

Om inzicht te krijgen welke eiwitten veranderd tot uiting komen hebben we de eiwitexpressie tussen DLBCL cellijnen en EBV geïnfecteerde B-cellen vergeleken (**Hoofdstuk 2**). Voor eiwitten met een verschil in expressie in de cellijnen werd vervolgens onderzoek verricht op tumorweefsel van DLBCL patiënten. In de cellijnen waren er 14 eiwitten met verminderde expressie, 8 eiwitten met toegenomen expressie en 10 met variabele expressie. Deze 32 eiwitten zijn betrokken bij cel bewegelijkheid, metabolisme, veranderingen van het chromatine, anti-oxidanten, immuunrespons en signaaloverdracht. De expressie van het anti-oxidatieve eiwit PRDX was hoger in DLBCL dan in folliculair lymfoom (een indolent lymfoom). PRDX kan de DLBCL cellen helpen om beter om te gaan met de hogere oxidatieve stress die deze cellen ervaren. Enigszins verrassend was ook de

expressie van PPIA, het aangrijpings punt van het immuun onderdrukkend medicijn ciclosporine toegenomen. In DLBCL cellijnen was ciclosporine in staat om de celgroei te remmen.

Een deel van de hooggradige lymfomen evolueert uit een folliculair lymfoom (FL). We laten zien dat in bijna 40% van de getransformeerde folliculaire lymfomen (tFL) een breuk aanwezig is in het *MYC*-gen (**Hoofdstuk 3**). Onze data ondersteunen de observatie dat MYC een sterke factor is voor transformatie van laaggradige lymfomen. In combinatie met observaties van anderen, lijken onze data erop te wijzen dat de fusie van het *MYC* gen met het IgH locus uniform leidt tot sterke expressie van het MYC eiwit, terwijl andere fusie partners leiden tot meer variabele MYC eiwit expressie. MYC eiwit expressie was significant hoger in de tFL dan in de gepaarde FL biopten. MYC eiwit expressie < 40% had in onze handen een uitstekende negatief voorspellende waarde. Dat wil zeggen dat het onwaarschijnlijk is dat casussen met een MYC eiwit expressie van < 40% een MYC breuk hebben.

Ongeacht het type agressief B-cel lymfoom worden deze alle gemerkt door veranderingen in eiwitten die normaal nodig zijn voor de herkenning door het afweersysteem (immune escape). In dit proefschrift laten we zien dat er frequent een defect is in de expressie van de humaan leukocyt antigenen (HLA) klasse I en klasse II eiwitten op het celoppervlak van tumorcellen (**Hoofdstuk 4**). We beschrijven een nieuw defect, namelijk verlies van HLA-DM. Hierdoor raakt het laden van antigenen in het HLA-II molecule verstoort en komt er een HLA-II molecule op het celoppervlak met altijd hetzelfde stukje eiwit (invariant chain). HLA verlies kan niet alleen een rol spelen bij het ontstaan van een B-NHL, maar heeft ook implicaties voor therapie. Inmiddels is duidelijk geworden dat bij patiënten met een Hodgkin lymfoom effectiviteit van immuuntherapie (checkpoint inhibitors) samenhangt met de aan- of afwezigheid van HLA-II. Hoewel een gestoorde antigeen expressie dus frequent voorkomt, verschilt het mechanisme per B-NHL type. Dit kan van belang zijn voor therapie die aangrijpt op HLA expressie. Recent is een relatie gelegd tussen HLA-I verlies en overexpressie van het *MYC* gen. We laten zien dat in DLBCL verlies van HLA-II frequenter wordt waargenomen in het ABC-type dan in het GCB-type (**Hoofdstuk 5**). Het idee is dat verlies van HLA-II een epigenetisch fenomeen is van B-cellen in de transitie naar plasma cellen. Immers, op normale plasma cellen komt geen HLA-II voor. Of manipulatie van HLA expressie mogelijk is en of dit functionele (d.w.z. therapeutische) consequenties heeft, dient verder onderzocht te worden.

Evolutie en therapieresistentie

De combinatie chemotherapie voor DLBCL met cyclofosfamide, doxorubicine, vincristine en prednisolon (CHOP) dateert uit 1976. Sinds 2000 is daar rituximab, een afweerstof gericht tegen het CD20 eiwit, aan toegevoegd. CD20 komt universeel voor op rijpe B-cellen. De afgelopen jaren zijn er verschillende medicijnen geregistreerd en beschikbaar gekomen voor patiënten met een laaggradig lymfoom. Helaas hebben deze medicijnen bij de behandeling van DLBCL de belofte van verbeterde overleving niet waar kunnen maken. Een van de uitdagingen is om de prognostische modellen te verbeteren, beter de heterogeniteit van DLBCL in kaart te brengen en de dynamiek (clonale evolutie) na R-CHOP maar ook gerichte therapie beter te doorgronden.

Van patiënten met een stadium I DLBCL wordt over het algemeen aangenomen dat ze een uitstekende prognose hebben. Recent heeft de Duitse lymfoom studiegroep data gepresenteerd over de-escalatie van R-CHOP binnen deze groep van patiënten. Wij laten in dit proefschrift zien, dat dit niet noodzakelijkwijs voor alle patiënten de optimale keuze is (**Hoofdstuk 6**). Wij onderzochten 126 patienten met een stadium I DLBCL en bestudeerden de groep (n=19) die een recidief kregen. Patiënten met een recidief hadden een slechte overleving. Dit had deels te maken met de ongunstige klinische en biologische factoren van deze patiënten, waaronder veranderingen in het *MYC*-gen en recidieven in de hersenen, factoren waar R-CHOP weinig invloed op heeft. De huidige klinische risico scores zijn niet in staat deze patiënten te identificeren.

Alhoewel het mutatie landschap van meer dan 2000 DLBCL samples inmiddels met nieuwe technieken (next generation sequencing) in kaart is gebracht, is er maar zeer beperkte informatie over de clonale evolutie van DLBCL. In dit proefschrift beschrijven we de bevindingen van onze pilotstudie naar veranderingen in het mutatieprofiel van gepaarde tumor samples (diagnose en recidief) (**Hoofdstuk 7**). Een van de uitdagingen waar we tegen aanliepen was het verkrijgen van tumormateriaal van voldoende kwaliteit om het hele coderende deel van het DNA (whole exome) in kaart te brengen. Dit lukte in 6 patiënten. In 4 van de 6 patiënten observeerden we een beperkte toename van mutaties. Dit lijkt ook in andere series het meest frequent voor te komen en past bij een lineaire evolutie vanuit een late kloon. Daarentegen, in de andere 2 patiënten waren de verschillen in mutaties groter. Dit kan passen binnen het model van clonale evolutie uit een vroege kloon met een divergent beloop. Bij onderzoek naar nieuwe therapieën is het goed om rekenschap te nemen van deze verschillen. Als een proof-of-concept is er één DLBCL patiënt beschreven die behandeld werd met een remmer van de bruton tyrosine kinase (BTK) en waarbij de klonen met de mutaties in BTK in de loop van de tijd de overhand kregen. Hierbij dient rekening gehouden te worden met het effect van AID, maar ook met mutaties die kunnen ontstaan door de therapie. In onze pilotstudie hadden 5 van de 6 patiënten een mutatie in PIM1 en SOCS1, waarvan ook anderen hebben beschreven dat ze gerelateerd zijn aan therapie resistentie. Tot

dusverre zijn er nog geen studies gepubliceerd over het effect van PIM-blokkers bij lymfomen, maar preklinische data lijkt te suggereren dat deze groep van remmers kankercellen gevoeliger kan maken voor chemotherapie.

Diagnostiek en behandeling

Ondanks dat de prognose voor patiënten met een laag risico DLBCL goed is, zijn de uitkomsten van patiënten met een hoog risico ziekte onbevredigend. De meest gebruikte risicoscore is de International Prognostic Index (IPI), waarbij de 2-jaars overleving varieert van 91% voor patiënten met een laag risico tot 59% voor patiënten met een hoog risico ziekte. Naast de IPI score kunnen patiënten die een verhoogd risico hebben op therapiefalen worden onderscheiden op basis van lokalisatie, ^{18}F-FDG PET scans tijdens behandeling, de cel-van-oorsprong, de aanwezigheid van breuken in het *MYC*-gen, overexpressie van eiwitten MYC en BCL2, mutaties in het DNA en veranderingen in de hoeveelheid circulerend tumor DNA (ctDNA) in het bloed. Echter, implementatie van deze 'nieuwe' factoren in de klinische risico-inschatting of behandelstrategie van patiënten heeft nog onvoldoende plaatsgevonden.

Hoewel het gebruik van PET-scans voor de stadiëring en responsbeoordeling van patiënten met een agressief B-NHL inmiddels standaard is, lijkt het voor prognose en interventies minder goed te functioneren. Dit kan ten dele komen doordat de brug tussen biologische eigenschappen van de tumor en PET-scans onvoldoende geïntegreerd onderzocht is. MYC geeft een hoge proliferatie en is daarmee een sterke aanjager van het metabolisme (**Hoofdstuk 3**). Daarom verrichtten wij een retrospectieve observationele studie naar de relatie tussen semi-kwantitatieve PET karakteristieken en necrose in DLBCL en HGBCL-DH (**Hoofdstuk 8**). We vonden geen verschil in de semi-kwantitatieve eigenschappen tussen de twee agressieve B-NHL. Wel constateerden we in 25% van de gevallen necrose, welke een negatief prognostische factor was voor ziektevrije overleving. Uiteraard moet men oppassen voor vals positieve resultaten bij de interpretatie van PET-scans (**Hoofdstuk 9**).

Behandeling van DLBCL is de laatste 20-jaar niet wezenlijk veranderd. Een belangrijk aspect is het risico op recidieven in de hersenen (**Hoofdstuk 6**). Hoge dosis methotrexaat (MTX) zou van toegevoegde waarde kunnen zijn in de profylactische behandelingen van hoog CNS risico patiënten, maar is nog niet bewezen effectiever dan toedieningen van MTX via een ruggenprik. In dit proefschrift laten we zien dat de combinatie van R-CHOP en hoge dosis MTX een effectieve therapie is voor patiënten met een agressief B-NHL met lokalisaties buiten en in het brein (**Hoofdstuk 10**). Hoewel intensieve chemotherapie gevolgd door een autologe stamceltransplantatie (ASCT) wordt gesuggereerd, is R-CHOP met MTX al toxisch. Wij en anderen toonden aan dat zonder ASCT een 2-jaars overleving van 40-70% bereikt kan worden.

Tenslotte beschrijven we de succesvolle behandeling van een patiënte met een therapieresistent PMBCL (**Hoofdstuk 11**). Op basis van een vermenigvuldiging van een stuk van chromosoom 9 komen de eiwitten program death ligand (PDL) en Janus Kinase 2 (JAK2) verhoogd tot expressie. In vitro is de combinatie van blokkers van beide eiwitten synergistisch. Monotherapie met pembrolizumab toonde bij een aanzienlijk deel van de patiënten afname van de tumorgrootte, maar het aantal patiënten met een complete remissie was gering. Patiënte werd succesvol behandeld met de nieuwe combinatie van pembrolizumab en ruxolitinib - een blokker van JAK2. Niet alleen bereikte patiënte een complete remissie, ook trad de respons binnen enkele weken op. Gezien het gunstig bijwerkingenprofiel van ruxolitinib is het een interessant medicament voor combinatie therapie.

Toekomstige ontwikkelingen

In het perspectief van bovenstaande kan gesteld worden dat er in de afgelopen decennia veel geleerd is over de biologie van agressieve B-cel lymfomen, maar dat dit maar een beperkte impact heeft gehad op de diagnostiek en behandeling. Confirmatie studies zijn nodig om de optimale biologische subclassificaties vast te stellen en relaties te leggen met klinische uitkomsten. Gezien de beperkingen in kwaliteit van biopten en de neiging om steeds minder invasieve diagnostiek te doen, lijkt de opkomst van analyse van circulerend tumor DNA (ctDNA) een grote stap voorwaarts. De combinatie van interim PET-scans en meting van minimale rest ziekte (MRD) op basis van ctDNA heeft de potentie om de heterogeniteit in DLBCL beter in kaart te brengen, de behandeling te sturen en clonale evolutie in kaart te brengen. Om echt stappen te maken, dienen we echter de Achilles hiel van DLBCL te vinden. Nieuwe behandelingen kunnen de inductietherapie helpen te verbeteren of als consolidatie worden gegeven in het geval van resterende minimale rest ziekte. De ontwikkeling van behandelingen kan verbeterd worden door slimmere studieontwerpen en door gedegen translationeel onderzoek, waaronder de mogelijkheid van moleculaire beeldvorming, welke de biodistributie van monoclonale antilichamen in kaart kan brengen. Dit kan bijdragen aan het selectieproces van patiënten die het meeste baat hebben bij de toekomstige behandelingen. Deze diagnostische en therapeutische overwegingen vormen de basis voor een deel van de vervolgstudies.

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